

REMARKS

Amendments

Claims 1-27, 33-36, 38-46 and 48-51 have been canceled, claims 28-32, 37, 47 and 52 have been amended, and claims 53-58 have been added. Upon entry of the amendment, claims 28-32, 37, 47 and 52-58 will be pending. Support for the amendments can be found in the specification, specifically on page 6, lines 28 through page 7, line 2; Example 1; the Figures, and in the claims as originally filed.

The foregoing amendments are made solely to expedite prosecution of the application and are not intended to limit the scope of the invention. Further, the amendments to the claims are made without prejudice to the pending or now canceled claims or to any subject matter pursued in a related application. The Applicant reserves the right to prosecute any canceled subject matter at a later time or in a later filed divisional, continuation, or continuation-in-part application.

Rejections

Rejections under 35 U.S.C. § 101

The Examiner has rejected claims 28-47 and 52 because the claimed invention is allegedly not supported by either a specific or substantial asserted utility or a well-established utility. (Although the Examiner has rejected the claims under 35 USC 112, para.1, the arguments set forth appear to be based on lack of utility. Applicant is therefore treating the Examiner's rejection as being under 101/112.)

Applicant respectfully traverses the rejection. Amended claim 1 is drawn to a transgenic mouse whose genome comprises a null endogenous PTP36 allele, wherein null allele comprises exogenous DNA. According to 35 U.S.C. § 101, "[w]hoever invents . . . any new and useful . . . composition of matter may obtain a patent therefore. . . ."

Under the Patent Office's Utility Requirement Guidelines:

If at any time during the examination, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility. An invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process), and (ii) the utility is specific, substantial, and credible.

. . .

If the applicant has asserted that the claimed invention is useful for any particular practical purpose (i.e., it has a “specific and substantial utility”) and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.

(emphasis added)(MPEP § 2107, II (A)(3); II (B)(1)).

The standard for “credible” is defined as:

. . . whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided. An assertion is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion.

(MPEP 2107.02, III(B)(emphasis added).

According to the Patent Office’s own guidance to Examiners:

Langer and subsequent cases direct the Office to presume that a statement of utility made by an applicant is true. [citations omitted] . . . Clearly, Office personnel should not begin an evaluation of utility by assuming that an asserted utility is likely to be false.

Compliance with 35 U.S.C. 101 is a question of fact [citations omitted]. Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, Office personnel must establish that it is more likely than not that one of ordinary skill in the art would doubt (i.e., “question”) the truth of the statement of utility. . . . To do this, Office personnel must provide evidence sufficient to show that the statement of asserted utility would be considered “false” by a person of ordinary skill in the art.

(MPEP 2107.02, III(A)(emphasis added).

Rejections under 35 U.S.C. 101 have been rarely sustained by federal courts.

Generally speaking, in these rare cases, the 35 U.S.C. 101 rejection was sustained either because the applicant failed to disclose any utility for the invention or asserted a utility that could only be true if it violated a scientific principle, such as the second law of thermodynamics, or a law of nature, or was wholly inconsistent with contemporary knowledge in the art. *In re Gazave*, 379 F.2d 973, 978, 154 USPQ 92, 96 (CCPA 1967). Special care therefore should be taken when assessing the credibility of an asserted therapeutic utility for a claimed invention. In such cases, a previous lack of success in treating a disease or condition, of the absence of a proven animal model for testing the effectiveness of drugs for treating a disorder in humans, should not, standing alone, serve as a basis for challenging the asserted utility under 35 U.S.C. 101.

(MPEP 2107.02, III(B)(emphasis in original and added). The Guidelines additionally provide that:

There is no predetermined amount or character of evidence that must be provided by an applicant to support an asserted utility, therapeutic or otherwise. Rather, the character and amount of evidence needed to support an asserted utility will vary depending on what is claimed (citations omitted), and whether the asserted utility appears to contravene established scientific principles and beliefs. (citations omitted). Furthermore, the applicant does not have to provide evidence sufficient to establish that an asserted utility is true “beyond a reasonable doubt.” (citations omitted). Nor must an applicant provide evidence such that it establishes an asserted utility as a matter of statistical certainty. Nelson v. Bowler, 626 F.2d 853, 856-57, 206 USPQ 881, 883-84 (CCPA 1980)(reversing the Board and rejecting Bowler’s arguments that the evidence of utility was statistically insignificant. The court pointed out that a rigorous correlation is not necessary when the test is reasonably predictive of the response).

(MPEP 2107.02, VII)(emphasis added).

Thus, according to Patent Office guidelines, a rejection for lack of utility may not be imposed where an invention has a well-established utility or is useful for any particular practical purpose. An assertion of utility is presumed to be true. The burden is on the Examiner to show that one of ordinary skill would find the asserted utility to be false. The present invention satisfies either standard.

The present invention has a well-established utility since a person of ordinary skill in the art “would immediately appreciate why” knockout mice are useful. As a general principle, knockout mice have the inherent and well-established utility of defining the function and role of the disrupted target gene, regardless of whether the inventor has described any specific phenotypes, characterizations or properties of the knockout mouse. The sequencing of the human genome has produced countless genes whose function has yet to be determined.

According to the National Institute of Health, knockout mice represent a critical tool in studying gene function:

Over the past century, the mouse has developed into the premier mammalian model system for genetic research. Scientists from a wide range of biomedical fields have gravitated to the mouse because of its close genetic and physiological similarities to humans, as well as the ease with which its genome can be manipulated and analyzed.

...

In recent decades, researchers have utilized an array of innovative genetic technologies to produce custom-made mouse models for a wide array of specific diseases, as well as to study the function of targeted genes. One of the most important advances has been the ability to create transgenic mice, in which a new gene is inserted into the animal's germline. Even more powerful approaches, dependent on homologous recombination, have permitted the development of

tools to "knock out" genes, which involves replacing existing genes with altered versions; or to "knock in" genes, which involves altering a mouse gene in its natural location. To preserve these extremely valuable strains of mice and to assist in the propagation of strains with poor reproduction, researchers have taken advantage of state-of-the-art reproductive technologies, including cryopreservation of embryos, in vitro fertilization and ovary transplantation.

(<http://www.genome.gov/pfv.cfm?pageid=10005834>)(emphasis added)(copy attached).

Thus, the knockout mouse has been accepted by the NIH as the premier model for determining gene function, a utility that is specific, substantial and credible.

Knockout mice are so well accepted as tools for determining gene function that the director of the NIH Chemical Genomics Center of the National Human Genome Research Institute (among others, including Capecchi, Bradley, Joyner, Nagy and Skarnes) has proposed creating knockout mice for all mouse genes:

Now that the human and mouse genome sequences are known, attention has turned to elucidating gene function and identifying gene products that might have therapeutic value. The laboratory mouse (*Mus musculus*) has had a prominent role in the study of human disease mechanisms throughout the rich, 100-year history of classical mouse genetics, exemplified by the lessons learned from naturally occurring mutants such as agouti, reeler and obese. The large-scale production and analysis of induced genetic mutations in worms, flies, zebrafish and mice have greatly accelerated the understanding of gene function in these organisms. Among the model organisms, the mouse offers particular advantages for the study of human biology and disease: (i) the mouse is a mammal, and its development, body plan, physiology, behavior and diseases have much in common with those of humans; (ii) almost all (99%) mouse genes have homologs in humans; and (iii) the mouse genome supports targeted mutagenesis in specific genes by homologous recombination in embryonic stem (ES) cells, allowing genes to be altered efficiently and precisely.

...

A coordinated project to systematically knock out all mouse genes is likely to be of enormous benefit to the research community, given the demonstrated power of knockout mice to elucidate gene function, the frequency of unpredicted phenotypes in knockout mice, the potential economies of scale in an organized and carefully planned project, and the high cost and lack of availability of knockout mice being made in current efforts.

(Austin et al., Nature Genetics (2004) 36(9):921-24, 921)(emphasis added)(copy attached).

With respect to claims drawn to transgenic mice having a null allele, the following comments from Austin are relevant:

Null-reporter alleles should be created

The project should generate alleles that are as uniform as possible, to allow efficient production and comparison of mouse phenotypes. The alleles should achieve a balance of utility, flexibility, throughput and cost. A null allele is an indispensable starting point for studying the function of every gene. Inserting a reporter gene (e.g., P-galactosidase or green fluorescent protein) allows a rapid assessment of which cell types normally support the expression of that gene.

(p. 922)(emphasis in original, emphasis added).

Research tools such as knockout mice are clearly patentable, as noted by the Patent

Office:

Some confusion can result when one attempts to label certain types of inventions as not being capable of having a specific and substantial utility based on the setting in which the invention is to be used. One example is inventions to be used in a research or laboratory setting. Many research tools such as gas chromatographs, screening assays, and nucleotide sequencing techniques have a clear, specific and unquestionable utility (e.g., they are useful in analyzing compounds). An assessment that focuses on whether an invention is useful only in a research setting thus does not address whether the invention is in fact “useful” in a patent sense. Instead, Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. Labels such as “research tool,” “intermediate” or “for research purposes” are not helpful in determining if an applicant has identified a specific and substantial utility for the invention.

(MPEP § 2107.01, I). As with gas chromatographs, screening assays and nucleotide sequencing techniques, knockout mice have a clear, specific and unquestionable utility (e.g., they are useful in analyzing gene function), one that is clearly recognized by those skilled in the art.

For example, according to the Molecular Biology of the Cell (Albert, 4th ed., Garland Science (2002)) (copy of relevant pages attached), one of the leading textbooks in the field of molecular biology:

Extensive collaborative efforts are underway to generate comprehensive libraries of mutation in several model organisms including . . . the mouse. The ultimate goal in each case is to produce a collection of mutant strains in which every gene in the organism has either been systematically deleted, or altered such that it can be conditionally disrupted. Collections of this type will provide an invaluable tool for investigating gene function on a genomic scale.

(p. 543)(emphasis added).

According to Genes VII (Lewin, Oxford University Press (2000)) (copy of relevant pages attached), another well respected textbook in the field of genetics:

The converse of the introduction of new genes is the ability to disrupt specific endogenous genes. Additional DNA can be introduced within a gene to prevent its expression and to generate a null allele. Breeding from an animal with a null allele can generate a homozygous “knockout”, which has no active copy of the gene. This is a powerful method to investigate directly the importance and function of the gene.

(p. 508)(emphasis added).

According to Joyner (Gene Targeting: *A Practical Approach*, Oxford University Press 2000) (copy of relevant pages attached),:

Gene targeting in ES cells offers a powerful approach to study gene function in a mammalian organism.

(preface)(emphasis added).

According to Matise et al. (*Production of Targeted Embryonic Stem Cell Clones* in Joyner, Gene Targeting: *A Practical Approach*, Oxford University Press 2000)(copy of relevant pages attached):

The discovery that cloned DNA introduced into tissue culture cells can undergo homologous recombination at specific chromosomal loci has revolutionized our ability to study gene function in cell culture and *in vivo*. . . . Thus, applying gene targeting technology to ES cells in culture affords researchers the opportunity to modify endogenous genes and study their function *in vivo*.

(p. 101)(emphasis added).

According to Crawley (What’s Wrong With My Mouse *Behavioral Phenotyping of Transgenic and Knockout Mice*, Wiley-Liss 2000) (copy of relevant pages attached):

Targeted gene mutation in mice represents a new technology that is revolutionizing biomedical research.

Transgenic and knockout mutations provide an important means for understanding gene function, as well as for developing therapies for genetic diseases.

(p. 1, rear cover)(emphasis added).

Applicant submits that since one of ordinary skill in the art would immediately recognize the utility of a knockout mouse in studying gene function, a utility that is specific, substantial and credible, the invention has a well-established utility, thus satisfying the utility requirement of

section 101. On this basis alone, withdrawal of the rejection with respect to the present invention is warranted, and respectfully requested.

In addition, the claimed invention is useful for a particular purpose. The Applicant has demonstrated and disclosed specific phenotypes of the presently claimed mice. Utility of the claimed knockout mouse would be apparent to, and considered credible by, one of skill in the art, as the role of knockout mice in studying any of these conditions is both specific and substantial.

The Examiner argues that the phenotypes do not correlate with human disease (current Office Action, p.5; April 27 Office Action, pp. 3-5). The Examiner's arguments are similar to arguments made by the Patent Office with respect to pharmaceutical compounds the utility of which were based on murine model data, arguments which were dismissed by the Federal Circuit in *In re Brana* (34 U.S.P.Q.2d 1436)(Fed. Cir. 1995). The case involved compounds that were disclosed to be effective as anti-tumor agents and had demonstrated activity against murine lymphocytic leukemias implanted in mice. The court ruled that the PTO had improperly rejected, for lack of utility, claims for pharmaceutical compounds used in cancer treatment in humans, since neither the nature of invention nor evidence proffered by the PTO would cause one of ordinary skill in art to reasonably doubt the asserted utility.

The first basis for the Board's holding of lack of utility (the Board adopted the examiner's reasoning without any additional independent analysis) was that the specification failed to describe any specific disease against which the claimed compounds were useful, and therefore, absent undue experimentation, one of ordinary skill in the art was precluded from using the invention. (*In re Brana* at 1439-40). The Federal Circuit reasoned that the leukemia cell lines were originally derived from lymphocytic leukemias in mice and therefore represented actual specific lymphocytic tumors. The court concluded that the mouse tumor models represented a specific disease against which the claimed compounds were alleged to be effective. (*In re Brana* at 1440).

The Board's second basis was that even if the specification did allege a specific use, the applicants failed to prove that the claimed compounds were useful.

The Federal Circuit responded: "[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of Section 112 unless there is

reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.” (*Brana* at 1441, citing *In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971)). From this it followed that the PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. (*Id.*)

The court held that the Patent Office had not met its burden. The references cited by the Board did not question the usefulness of any compound as an antitumor agent or provide any other evidence to cause one of skill in the art to question the asserted utility of applicants' compounds. Rather, the references merely discussed the therapeutic predictive value of *in vivo* murine tests -- relevant only if the applicants were required to prove the ultimate value in humans of their asserted utility. The court did not find that the nature of the invention alone would cause one of skill in the art to reasonably doubt the asserted usefulness. The purpose of treating cancer with chemical compounds did not suggest an inherently unbelievable undertaking or involve implausible scientific principles. (*Id.*)

The Court concluded that one skilled in the art would be without basis to reasonably doubt the asserted utility on its face. The PTO had not satisfied its initial burden. Accordingly, the applicants should not have been required to substantiate their presumptively correct disclosure to avoid a rejection under the first paragraph of Section 112. (*Id.*)

As in *Brana*, Applicant has asserted that the claimed invention is useful for a particular practical purpose, an assertion that would be considered credible by a person of ordinary skill in the art. As discussed above, the claimed mice have demonstrated specific phenotypes. The acceptance among those of skill in the art of knockout mice demonstrating such properties is clearly demonstrated.

Definitive proof that the phenotypes observed in the null mouse would be the same as those observed in humans is not a prerequisite to satisfying the utility requirement. It is enough that the claimed mouse demonstrates phenotypes, relative to a wild type control mouse, and that knockout mice are recognized in the art as models for determining gene function, both in mice and in humans. According to Austin et al.:

Among the model organisms, the mouse offers particular advantages for the study of human biology and disease: (i) the mouse is a mammal, and its development, body plan, physiology, behavior and diseases have much in common with those of humans; (ii) almost all (99%) mouse genes have homologs in humans; and (iii) the mouse genome supports targeted mutagenesis in specific genes by homologous recombination in embryonic stem (ES) cells, allowing genes to be altered efficiently and precisely.

(p. 921)(emphasis added).

In addition, as pointed out by Doetschman, one clearly skilled in the art, (*Laboratory Animal Science* 49:137-143, 137 (1999)(copy attached), the phenotypes observed in mice do correlate to gene function:

The conclusions will be that the knockout phenotypes do, in fact, provide accurate information concerning gene function, that we should let the unexpected phenotypes lead us to the specific cell, tissue, organ culture, and whole animal experiments that are relevant to the function of the genes in question, and that the absence of phenotype indicates that we have not discovered where or how to look for a phenotype.

(emphasis added).

In *Brana*, the claimed compound had demonstrated activity against a murine tumor implanted in a mouse. Yet, the Federal Circuit found that utility had been demonstrated. Here, the invention relates to a disruption in a murine gene in a mouse. Like the tumor mouse model, the knockout mouse with a specific gene disrupted is a widely accepted model, the utility of which would be readily accepted in the art. It is submitted that one skilled in the art would be without basis to be reasonably doubt Applicant's asserted utility, and therefore the Examiner has not satisfied the initial burden.

The Examiner argues that the asserted utility is not specific to the PTP36 knockout mouse.

Applicant disagrees. According to the MPEP, "specific utility" means "specific" to the subject matter claimed as compared to a "general utility" that would be applicable to the broad class of the invention (MPEP 2107.01). Use of the GAL1R $-/+$ mouse to study the function of the GAL1R gene and the association of the GAL1R gene with the development of the female sex organs is specific to this mouse. Even if there were many other genes associated with sexual development, only a GAL1R knockout mouse (as opposed to all other knockout mice) would be used to study the specific role of GAL1R.

The Examiner argues that the asserted use to study gene function is an invitation for further research.

According to the MPEP, under the section entitled "Substantial Utility,"

A "substantial utility" defines a "real world" use. Utilities that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use are not substantial utilities. . . . the following are examples of situations that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use and, therefore, do not define "substantial utilities":

(A) Basic research such as studying the properties of the claimed product itself or the mechanisms in which the material is involved;

Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations in other cases to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. See, e.g., Brenner v. Manson, 383 U.S. 519, 534-35, 148 USPQ 689, 695 (1966). Rather, **any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a "substantial" utility.**

(MPEP § 2107.01 I)(emphasis added).

The MPEP additionally provides:

Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. Labels such as "research tool," "intermediate" or "for research purposes" are not helpful in determining if an applicant has identified a specific and substantial utility for the invention.

(MPEP § 2107.01, I). As set forth in the above-cited portions of the MPEP guidelines, a use is not substantial where further research is required to identify any use. Such is not the case here. Knockout mice have a well-known use to study gene function. In the present case, the present invention does not require further research to establish any utility. Applicant has determined that the GRL1R gene is associated with, for example, sex organ development. The Applicant has provided an immediate benefit to the public. No further research is required to establish a use. Whether additional research is required to identify drugs capable of targeting the GRL1R receptor or gene or to further investigate the function of the gene is irrelevant to whether the claimed invention has satisfied the utility requirement.

With regard to the section 103 rejections on obviousness, the Applicant maintains his view that the Examiner has effectively admitted that one skilled in the art would know how to make and use the claimed invention. It is contradictory to argue that one of ordinary skill would have been motivated to make and use a knockout mouse but then argue that such a mouse once created would not meet the utility requirement.

The Examiner argues that *Brana* does apply to the instant case, and that the Applicant has taken one conclusion out of context.

Applicant disagrees. The “further research and development” referred to in *Brana* was the PTO’s unreasonable requirement that the drug be shown to be effective in humans in order to satisfy the utility requirement. Here, the Examiner is arguing that since additional research is required, the claimed invention lacks utility. However, as set forth above in the Utility Guidelines, “any” reasonable asserted use should be regarded as substantial.

The Applicant submits that the legal principles as well as the facts of *Brana* are applicable to the present case. In *Brana*, the Board held that the applicant’s specification failed to disclose a specific disease against which the claimed compounds were useful. The Federal Circuit reversed and held that the mouse tumor model represented a specific disease against which the compounds were effective. In the present case, the Examiner has argued that the mice lack utility as they allegedly are not a model for any human disease (page 5). It is Applicant’s position that a mouse demonstrating sex organ abnormalities and growth abnormalities is sufficient to establish the animal’s use as a model of a disease or condition. Confirmation of the phenotype in humans is unnecessary.

In *Brana*, the PTO did not consider the asserted use to satisfy the utility requirement:

Applicants' specification, however, also states that the claimed compounds have "a better action and a better action spectrum as antitumor substances" than known compounds, specifically those analyzed in Paull. As previously noted, see supra note 4, Paull grouped various benzo [de]isoquinoline-1,3-diones, which had previously been tested in vivo for antitumor activity against two lymphocytic leukemia tumor models (P388 and L1210), into various structural classifications and analyzed the test results of the groups (i.e. what percent of the compounds in the particular group showed success against the tumor models). Since one of the tested compounds, NSC 308847, was found to be highly effective against these two lymphocytic leukemia tumor models, 14 applicants' favorable comparison implicitly asserts that their claimed compounds are highly effective (i.e. useful) against lymphocytic leukemia. An alleged use against this particular type of cancer is much more specific than the vaguely intimated uses rejected by the courts in *Kirk* and *Kawai*. See, e.g., *Cross v. Iizuka*, 753 F.2d at 1048, 224

USPQ at 745 (finding the disclosed practical utility for the claimed compounds -- the inhibition of thromboxane synthetase in human or bovine platelet microsomes -- sufficiently specific to satisfy the threshold requirement in Kirk and Kawai.)

The Commissioner contends, however, that P388 and L1210 are not diseases since the only way an animal can get sick from P388 is by a direct injection of the cell line. The Commissioner therefore concludes that applicants' reference to Paull in their specification does not provide a specific disease against which the claimed compounds can be used. We disagree.

(Brana at 1440). Thus, the PTO was aware of the asserted use against the mouse tumor lines but did not find the use specific – as in the present case.

An assertion of utility is presumed to be true. The burden is on the Examiner to show that one of ordinary skill would find the asserted utility to be false. The Examiner has apparently ignored the Utility Guidelines by presuming the asserted utility to be false (“Clearly, Office personnel should not begin an evaluation of utility by assuming that an asserted utility is likely to be false.” MPEP 2107.02)

According to the MPEP, if an “assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility” (MPEP § 2107, II (A)(3); II (B)(1)). If it is well known to those skilled in the art that KO mice are useful for studying gene function, then those skilled in the art would certainly regard such use as credible. Nothing more is required to satisfy the statutory requirement.

Applicant submits that whether further research is required does not make the asserted use any less credible, substantial or specific. The Applicant has satisfied the requirements set forth in the MPEP guidelines: that one skilled in the art would regard the asserted utility as credible.

In addition to their use in studying gene function, the claimed transgenic mice are useful for studying gene expression. The mice within the scope of the amended claims contain a visible marker such as lacZ. Their use in studying gene expression is clearly recognized by those skilled in the art:

Null-reporter alleles should be created

The project should generate alleles that are as uniform as possible, to allow efficient production and comparison of mouse phenotypes. The alleles should achieve a balance of utility, flexibility, throughput and cost. A null allele is an indispensable starting point for studying the function of every gene. Inserting a reporter gene (e.g., P-galactosidase

or green fluorescent protein) allows a rapid assessment of which cell types normally support the expression of that gene.

(Austin et al., Nature Genetics (2004) 36(9):921-24, 922)(emphasis in original; emphasis added)(copy attached). Applicant respectfully reminds Examiner that a claimed invention need only satisfy one of its stated objectives to satisfy the utility and enablement requirements.

In summary, Applicant submits that the claimed transgenic mouse, regardless of any disclosed phenotypes, has inherent and well-established utility in the study of the function of the gene, and thus satisfies the utility requirement of section 101. Moreover, Applicant believes that the transgenic mice are useful for studying GAL1R gene function with respect to the cited phenotypes, for studying gene expression, and are therefore useful for a specific practical purpose that would be readily understood by and considered credible by one of ordinary skill in the art.

In light of the arguments set forth above, Applicant does not believe that the Examiner has properly made a *prima facie* showing that establishes that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the Applicant to be specific and substantial. (*In re Brana*; MPEP § 2107).

Withdrawal of the rejections is respectfully requested.

Rejection under 35 U.S.C. § 112, first paragraph

The Examiner has rejected claims 28-47 and 52 because one skilled in the art would allegedly not know how to use the claimed invention as a result of the alleged lack of either a specific or substantial asserted utility or a well-established utility for the reasons set forth in the utility rejection. Applicants respectfully traverse the rejection. For the reasons set forth above, the claimed invention satisfies the utility requirement. Therefore, one skilled in the art would know how to use the claimed invention – a mouse having a null GALR1 allele.

Rejection under 35 U.S.C. § 112, second paragraph

The Examiner has rejected claim 52 “for the reasons set forth below” (page 2).

However, there are no arguments set forth in the body of the Office Action with regard to such rejection. Elaboration or withdrawal is respectfully requested.

In view of the above amendments and remarks, Applicant respectfully requests a Notice of Allowance. If the Examiner believes a telephone conference would advance the prosecution of this application, the Examiner is invited to telephone the undersigned at the below-listed telephone number.

The Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. 13-2725.

Respectfully submitted,

2-17-05
Date



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URL of this page: <http://www.genome.gov/10005834>

Background on Mouse as a Model Organism

Over the past century, the mouse has developed into the premier mammalian model system for genetic research. Scientists from a wide range of biomedical fields have gravitated to the mouse because of its close genetic and physiological similarities to humans, as well as the ease with which its genome can be manipulated and analyzed.

Although yeasts, worms and flies are excellent models for studying the cell cycle and many developmental processes, mice are far better tools for probing the immune, endocrine, nervous, cardiovascular, skeletal and other complex physiological systems that mammals share. Like humans and many other mammals, mice naturally develop diseases that affect these systems, including cancer, atherosclerosis, hypertension, diabetes, osteoporosis and glaucoma. In addition, certain diseases that afflict humans but normally do not strike mice, such as cystic fibrosis and Alzheimer's, can be induced by manipulating the mouse genome and environment. Adding to the mouse's appeal as a model for biomedical research is the animal's relatively low cost of maintenance and its ability to quickly multiply, reproducing as often as every nine weeks.

Mouse models currently available for genetic research include thousands of unique inbred strains and genetically engineered mutants. There are mice prone to different cancers, diabetes, obesity, blindness, Lou Gehrig's disease, Huntington's disease, anxiety, aggressive behavior, alcoholism and even drug addiction. Immunodeficient mice can also be used as hosts to grow both normal and diseased human tissue, a boon for cancer and AIDS research.

In the early days of biomedical research, scientists developed mouse models by selecting and breeding mice to produce offspring with the desired traits. Researchers also learned to produce useful, new models of genetic disease quickly and in large numbers by exposing mice to DNA-damaging chemicals, a process known as chemical mutagenesis.

In recent decades, researchers have utilized an array of innovative genetic technologies to produce custom-made mouse models for a wide array of specific diseases, as well as to study the function of targeted genes. One of the most important advances has been the ability to create transgenic mice, in which a new gene is inserted into the animal's germline. Even more powerful approaches, dependent on homologous recombination, have permitted the development of tools to "knock out" genes, which involves replacing existing genes with altered versions; or to "knock in" genes, which involves altering a mouse gene in its natural location. To preserve these extremely valuable strains of mice and to assist in the propagation of strains with poor reproduction, researchers have taken advantage of state-of-the-art reproductive technologies, including cryopreservation of embryos, *in vitro* fertilization and ovary transplantation.

The Jackson Laboratory, a publicly supported national repository for mouse models in Bar Harbor, Maine, has played a crucial role in the development of the mouse into the leading model for biomedical research. Established in 1929, the non-profit center pioneered the use of inbred laboratory mice to uncover the genetic basis of human development and disease. In fact, the famous "Black 6" or C57BL/6J mouse strain whose genome is the focus of the landmark sequencing effort was

developed in the early 1920s by The Jackson Laboratory founder Clarence Cook Little.

Today, researchers at The Jackson Laboratory pursue projects in areas that include cancer, development and aging, immune system and blood disorders, neurological and sensory disorders, and metabolic diseases. Informatics researchers work with the public sequencing consortium to curate and integrate the sequenced mouse genome data with the wealth of biological knowledge collected in Jackson's Mouse Genome Informatics resource.

In addition, The Jackson Laboratory distributes 2,700 different strains and stocks as breeding mice, frozen embryos or DNA samples. In FY 2002 alone, the lab supplied approximately 2 million mice to the international scientific community.

Listed below is a sampling of mouse models developed and/or distributed by The Jackson Laboratory, along with brief descriptions of the human diseases they are helping scientists to understand:

- Down Syndrome - One of the most common genetic birth defects in humans, occurring once in every 800 to 1,000 live births, Down syndrome results from an extra copy of chromosome 21, an abnormality known as trisomy. The Ts65Dn mouse, developed at The Jackson Laboratory, mimics trisomy 21 and exhibits many of the behavioral, learning, and physiological defects associated with the syndrome in humans, including mental deficits, small size, obesity, hydrocephalus and thymic defects. This model represents the latest and best improvement of Down syndrome models to facilitate research into the human condition.

- Cystic Fibrosis (CF) - The *Cftr* knockout mouse has helped advance research into cystic fibrosis, the most common fatal genetic disease in the United States today, occurring in approximately one of every 3,300 live births. Scientists now know that CF is caused by a small defect in the gene that manufactures CFTR, a protein that regulates the passage of salts and water in and out of cells. Studies with the *Cftr* knockout have shown that the disease results from a failure to clear certain bacteria from the lung, which leads to mucus retention and subsequent lung disease. These mice have become models for developing new approaches to correct the CF defect and cure the disease.

- Cancer - The p53 knockout mouse has a disabled *Trp53* tumor suppressor gene that makes it highly susceptible to various cancers, including lymphomas and osteosarcomas. The mouse has emerged as an important model for human Li-Fraumeni syndrome, a form of familial breast cancer.

- Glaucoma - The DBA/2J mouse exhibits many of the symptoms that are often associated with human glaucoma, including elevated intraocular pressure. Glaucoma is a debilitating eye disease that is the second leading cause of blindness in the United States.

- Type 1 Diabetes - This autoimmune disease, also known as Juvenile Diabetes, or Insulin Dependent Diabetes Mellitus (IDDM), accounts for up to 10 percent of diabetes cases. Non-obese Diabetic (NOD) mice are enabling researchers to identify IDDM susceptibility genes and disease mechanisms.

- Type 2 Diabetes - A metabolic disorder also called Non-Insulin Dependent Diabetes Mellitus (NIDDM), this is the most common form of diabetes and occurs primarily after age 40. The leading mouse models for NIDDM and obesity research were all developed at The Jackson Laboratory: *Cpe^{fat}*, *Lep^{ob}*, *Lepr^{db}* and *tub*.

- Epilepsy - The "slow-wave epilepsy," or *swe*, mouse is the only model to exhibit both of the two major forms of epilepsy: petit mal (absence) and grand mal (convulsive). It shows particular promise for research into absence seizures, which occur most often in children.

- Heart Disease - Elevated blood cholesterol levels and plaque buildup in arteries within

three months of birth (even on a low-fat diet) are characteristics of several experimental models for human atherosclerosis: the *Apoe* knockout mouse and C57BL/6J.

- Muscular Dystrophy - The *Dmd*^{mdx} mouse is a model for Duchenne Muscular Dystrophy, a rare neuromuscular disorder in young males that is inherited as an X-linked recessive trait and results in progressive muscle degeneration.

- Ovarian Tumors - The SWR and SWXJ mouse models provide excellent research platforms for studying the genetic basis of ovarian granulosa cell tumors, a common and very serious form of malignant ovarian tumor in young girls and post-menopausal women.

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The Knockout Mouse Project

Mouse knockout technology provides a powerful means of elucidating gene function *in vivo*, and a publicly available genome-wide collection of mouse knockouts would be significantly enabling for biomedical discovery. To date, published knockouts exist for only about 10% of mouse genes. Furthermore, many of these are limited in utility because they have not been made or phenotyped in standardized ways, and many are not freely available to researchers. It is time to harness new technologies and efficiencies of production to mount a high-throughput international effort to produce and phenotype knockouts for all mouse genes, and place these resources into the public domain.

Now that the human and mouse genome sequences are known¹⁻³, attention has turned to elucidating gene function and identifying gene products that might have therapeutic value. The laboratory mouse (*Mus musculus*) has had a prominent role in the study of human disease mechanisms throughout the rich, 100-year history of classical mouse genetics, exemplified by the lessons learned from naturally occurring mutants such as *agouti*⁴, *reeler*⁵ and *obese*⁶. The large-scale production and analysis of induced genetic mutations in worms, flies, zebrafish and mice have greatly accelerated the understanding of gene function in these organisms. Among the model organisms, the mouse offers particular advantages for the study of human biology and disease: (i) the mouse is a mammal, and its development, body plan, physiology, behavior and diseases have much in common with those of humans; (ii) almost all (99%) mouse genes have homologs in humans; and (iii) the mouse genome supports targeted mutagenesis in specific genes by homologous recombination in embryonic stem (ES) cells, allowing genes to be altered efficiently and precisely.

The ability to disrupt, or knock out, a specific gene in ES cells and mice was developed in the late 1980s (ref. 7), and the use of knockout mice has led to many insights into human biology and disease⁸⁻¹¹. Current technology also permits insertion of 'reporter' genes into the knocked-out gene, which can then be used to determine the temporal and spatial

expression pattern of the knocked-out gene in mouse tissues. Such marking of cells by a reporter gene facilitates the identification of new cell types according to their gene expression patterns and allows further characterization of marked tissues and single cells.

Appreciation of the power of mouse genetics to inform the study of mammalian physiology and disease, coupled with the advent of the mouse genome sequence and the ease of producing mutated alleles, has catalyzed public and private sector initiatives to produce mouse mutants on a large scale, with the goal of eventually knocking out a substantial portion of the mouse genome^{12,13}. Large-scale, publicly funded gene-trap programs have been initiated in several countries, with the International Gene Trap Consortium coordinating certain efforts and resources¹⁴⁻¹⁷.

Despite these efforts, the total number of knockout mice described in the literature is relatively modest, corresponding to only ~10% of the ~25,000 mouse genes. The curated Mouse Knockout & Mutation Database lists 2,669 unique genes (C. Rathbone, personal communication), the curated Mouse Genome Database lists 2,847 unique genes, and an analysis at Lexicon Genetics identified 2,492 unique genes (H.Z., unpublished data). Most of these knockouts are not readily available to scientists who may want to use them in their research; for example, only 415 unique genes are represented as targeted mutations in the Jackson Laboratory's Induced Mutant Resource database (S. Rockwood, personal communication).

The converging interests of multiple members of the genomics community led to a meeting to discuss the advisability and feasibility of

a dedicated project to produce knockout alleles for all mouse genes and place them into the public domain. The meeting took place from 30 September to 1 October 2003 at the Banbury Conference Center at Cold Spring Harbor Laboratory. The attendees of the meeting are the authors of this paper.

Is a systematic project warranted?

A coordinated project to systematically knock out all mouse genes is likely to be of enormous benefit to the research community, given the demonstrated power of knockout mice to elucidate gene function, the frequency of unpredicted phenotypes in knockout mice, the potential economies of scale in an organized and carefully planned project, and the high cost and lack of availability of knockout mice being made in current efforts. Moreover, implementing such a systematic and comprehensive plan will greatly accelerate the translation of genome sequences into biological insights. Knockout ES cells and mice currently available from the public and private sectors should be incorporated into the genome-wide initiative as much as possible, although some may need to be produced again if they were made with suboptimal methods (e.g., not including a marker) or if their use is restricted by intellectual property or other constraints. The advantages of such a systematic and coordinated effort include efficient production with reduced costs; uniform use of knockout methods, allowing for more comparability between knockout mice; and ready access to mice, their derivatives and data to all researchers without encumbrance. Solutions to the logistical, organizational and informatics issues associated with producing, characterizing and distributing such a large number of

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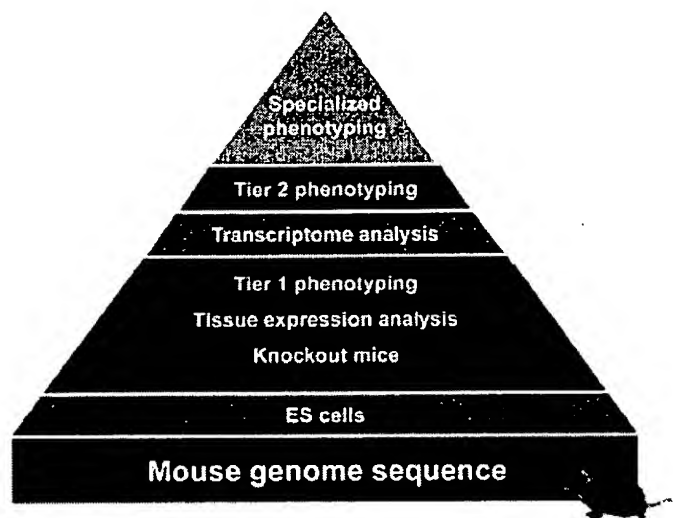


Figure 1 Structure of resource production in the proposed KOMP. Using the mouse genome sequence as a foundation, knockout alleles in ES cells will be produced for all genes. A subset of ES cell knockouts will be used each year to produce knockout mice, determine the expression pattern of the targeted gene in a variety of tissues and carry out screening-level (Tier 1) phenotyping. In a subset of mouse lines, transcriptome analysis and more detailed system-specific (Tier 2) phenotyping will be done. Finally, specialized phenotyping will be done on a smaller number of mouse lines with particularly interesting phenotypes. All stages will occur within the purview of the KOMP except for the specialized phenotyping, which will occur in individual laboratories with particular expertise.

mice will draw from the experience of related projects in the private sector and in academia, which have made or phenotyped hundreds of knockout mice using a variety of techniques. Lessons learned from these projects include the need for redundancy at each step to mitigate pipeline bottlenecks and the need for robust informatics systems to track the production, analysis, maintenance and distribution of thousands of targeting constructs, ES cells and mice.

Null-reporter alleles should be created

The project should generate alleles that are as uniform as possible, to allow efficient production and comparison of mouse phenotypes. The alleles should achieve a balance of utility, flexibility, throughput and cost. A null allele is an indispensable starting point for studying the function of every gene. Inserting a reporter gene (e.g., β -galactosidase or green fluorescent protein) allows a rapid assessment of which cell types normally support the expression of that gene. Therefore, we propose to produce a null-reporter allele for each gene. Making each mutation conditional in nature by adding *cis*-elements (e.g., *loxP* or FRT sites) would

be desirable, but we do not advocate this as part of the mutagenesis strategy unless the technological limitations currently associated with generating conditional targeted mutations on a large scale and in a cost-effective manner can be overcome.

A combination of methods should be used. Various methods can be used to create mutated alleles, including gene targeting, gene trapping and RNA interference. Advantages of conventional gene targeting include flexibility in design of alleles, lack of limitation to integration hot spots, reliability for producing complete loss-of-function alleles, ability to produce reporter knock-ins and conditional alleles, and ability to target splice variants and alternative promoters. BAC-based targeting has the potential advantages of higher recombination efficiencies and flexibility for producing complex mutated alleles¹⁸. Gene trapping is rapid, is cost-effective and produces a large variety of insertional mutations throughout the genome but can be somewhat less flexible^{17,19–21}. There is uncertainty regarding the percentage of gene traps that produce a true null allele and the fraction

of the genome that can ultimately be covered by gene-trap mutations. Trapping is not entirely random but shows preference for larger transcription units and genes more highly expressed in ES cells. In recent studies, gene trapping was estimated to potentially produce null alleles for 50–60% of all genes, perhaps more if a variety of gene-trap vectors with different insertion characteristics is used^{17,21}. RNA interference offers enormous promise for analysis of gene function in mice²² but is not yet sufficiently developed for large-scale production of gene modifications capable of reliably producing true null alleles. Both gene-targeting and gene-trapping methods are suitable for producing large numbers of knockout alleles, and, given their complementary advantages, a combination of these methods should be used to produce the genome-wide collection of null-reporter alleles most efficiently.

What should the deliverables be?

A genome-wide knockout mouse project could deliver to the research community a trove of valuable reagents and data, including targeting and trapping constructs and vectors, mutant ES cell lines, live mice, frozen sperm, frozen embryos, phenotypic data at a variety of levels and detail, and a database with data visualization and mining tools. At a minimum, we believe that a comprehensive genome-wide resource of mutant ES cell lines from an inbred strain, each with a different gene knocked out, should be produced and made available to the community. Choosing an inbred line (129/SvEvTac or C57BL/6J), and evaluating the alternative of using F₁ ES cells and tetraploid aggregation to provide potential time savings, merits additional scientific review and discussion^{23,24}. ES cells should be converted into mice at a rate consistent with project funding and the ability of the worldwide scientific community to analyze them. Although the value and cost-effectiveness of systematically characterizing the mice is a matter of debate, a limited set of broad and cost-effective screens, probably including assessment of developmental lethality, physical examination, basic blood tests, and histochemical analysis of reporter gene expression, would be useful. More detailed phenotyping, based on findings from the initial screen or existing knowledge of the gene's function, could be done at specialized centers. All ES cell clones and mice (as frozen embryos or sperm) should be available to any researcher at minimal cost, and all mouse phenotyping and reporter expression data should be deposited into a public database.

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In determining how to implement the project, utility to the research community should be the standard for judging value. Each step after ES cell generation (e.g., mouse creation, breeding, expression analysis, phenotyping) will make the resource useful to more researchers but will also increase costs and scientific complexity. We therefore advocate a 'pyramid' structure for the project (Fig. 1). At the base of the pyramid is the genome-wide collection of mutant ES cells for every mouse gene. Over time, a subset of these mutant ES cells should be made into mice and characterized with an initial phenotype screen (Tier 1; Fig. 1) and analysis of tissue reporter-gene expression. A subset of these lines should be profiled by microarray analysis, and a subset of these profiled by system-specific (Tier 2) phenotyping, based on the results of the Tier 1 phenotyping, array studies, existing knowledge of the gene's function and the gene's tissue expression pattern. With time, the upper tiers of the pyramid will be filled out, eventually transforming the pyramid into a cube, with information of all types available for all genes.

This project will require the resolution of numerous intellectual property claims involving the production and use of knockout mice. To deal with the existing patents that cover the technologies and processes involved in the production of mutant mice, we suggest that a 'patent pool', such as that used in the semiconductor industry²³, should be generated. Several individuals who represent entities that control patents on mouse knockout technologies are authors on this paper, and they agree with this approach. We also agree that any mutant ES cells or mice produced should be placed immediately in the public domain.

Mechanisms and costs

ES cell production. Automated knockout construct and ES cell production should be carried out in coordinated centers to ensure efficiency and uniformity. We estimate that most known mouse genes could be knocked out in ES cells within 5 years, using a combination of gene-trapping and gene-targeting techniques. Gene trapping can produce a large number of mutated alleles quickly, but its progress should be monitored closely to determine when its yield of new genes diminishes¹⁷ and, therefore, when targeting should be increasingly relied on. As large-scale trapping projects have already defined gene classes that probably cannot be knocked out by trapping (e.g., single-exon GPCRs, genes that are not expressed in ES cells), we propose that targeting begin on those classes immediately. All ES cells should be made available to the research community, because this collection itself

would be a valuable resource. Efforts in the public and private sectors have already knocked out many genes in ES cells, and, to the degree that the alleles produced fit the prescribed characteristics (i.e., null alleles with a reporter) and are available, every effort should be made to incorporate these into the planned public resource. Costs for generating this part of the resource were estimated at between \$9–11 million/year for five years (these and all subsequent figures are direct costs).

Mouse production. The subset of ES cells made into mice each year should be chosen by a peer-review process. Central facilities for high-efficiency mouse production, genotyping, breeding, maintenance and archiving should be funded, to take advantage of efficiencies of scale in mouse creation and distribution. Researchers could apply to produce groups of mice outside the centers, as long as they meet the cost specifications of the program. All mice should be made available immediately to researchers as frozen embryos or sperm, for nominal distribution cost. An initial target of 500 new mouse lines per year would double the current rate at which new genes are knocked out in the public sector; we feel that this rate is within the capacity of the biomedical research community worldwide to absorb and analyze. We estimated the initial cost of this level of mouse production to be \$12.5–15 million per year.

Reporter tissue expression analysis. Approximately 30 tissues from adult and developmental stages should be sampled to cover the main organ systems. Analysis methods should be customized to the organ system and marker, and a searchable database of the sites of gene expression, and the images showing them, should be produced. Centers to carry out these analyses and data curation should be selected by peer review. We estimated the cost of this component for 500 mouse lines to be \$2.5–5 million per year, depending on how much tissue sectioning and cell-level analysis is done.

Phenotyping. Tier 1 phenotyping should be a low-cost screen for clear phenotypes and should be done on all mouse lines produced. Tier 1 should include home-cage observation, physical examination, blood hematological and chemistry profiles, and skeletal radiographs. The centers producing the mice should carry out the Tier 1 analyses, at an estimated cost of \$2.5 million per year for 500 lines. Selected lines, chosen on the basis of findings from Tier 1 phenotyping, tissue expression patterns, microarray data and the scientific literature, should undergo more detailed and system-focused Tier 2 phenotyping. Tier 2 phenotyping should be done in

specialized phenotyping centers, akin to those already in operation for phenotyping of mice produced by ENU mutagenesis. All Tier 1 and Tier 2 phenotyping should be done on a uniform genetic background by dedicated groups of individuals in single locations, to facilitate consistency and cross-comparison of results among different mouse lines. All Tier 1 and Tier 2 phenotyping results should be deposited into a central project database freely accessible to the research community. More detailed and specialized phenotyping could be done by individual researchers in their own laboratories; deposition of this more detailed phenotype data would be encouraged.

Transcriptome analysis. Transcriptome profiling of tissues from each knockout line, collected in a uniform way across all mice and tissues and placed into a searchable relational database, would add substantially to the scientific value of the project, though it would also add considerably to its cost. Transcriptome analysis should therefore be done on a subset of mice, chosen by peer review. We estimate that, with the best currently available array technology, an analysis of ten tissues would cost ~\$18,000 per line.

Conclusions

This project, tentatively named the Knockout Mouse Project (KOMP), will be a crucial step in harnessing the power of the genome to drive biomedical discovery. By creating a publicly available resource of knockout mice and phenotypic data, KOMP will knock down barriers for biologists to use mouse genetics in their research. The scientific consensus that we achieved—that a dedicated project should be undertaken to produce mutant mice for all genes and place them into the public domain—is important but is only the beginning. Implementation of these recommendations will require additional input from the greater scientific community, including those responsible for programmatic direction and financial support of biomedical research in the public and private sectors. This ambitious and historic initiative must be carried out as a collaborative effort of the worldwide scientific community, so that all can contribute their skills, and all can benefit. International discussions among scientific and programmatic staffs since the Banbury meeting at Cold Spring Harbor, in both the public and private sectors, have shown that there is great enthusiasm and commitment to this vision. The next step for KOMP will be to move this visionary plan from conceptualization to implementation, with an urgency befitting the benefits it will bring to science and medicine.

COMMENTARY

URLs. The curated Mouse Knockout & Mutation Database is available at <http://research.bmn.com/mkmdl>. The curated Mouse Genome Database is available at <http://www.informatics.jax.org/>. *Patent pools: A solution to the problem of access in biotechnology patents?* is available at <http://www.uspto.gov/web/offices/pat/dapp/opla/patentpool.pdf>.

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Special Topic Overview

Interpretation of Phenotype in Genetically Engineered Mice

Thomas Doetschman

Background and Purpose: In mice, genetic engineering involves two general approaches—addition of an exogenous gene, resulting in transgenic mice, and use of knockout mice, which have a targeted mutation of an endogenous gene. The advantages of these approaches is that questions can be asked about the function of a particular gene in a living mammalian organism, taking into account interactions among cells, tissues, and organs under normal, disease, injury, and stress situations.

Methods: Review of the literature concentrating principally on knockout mice and questions of unexpected phenotypes, lack of phenotype, redundancy, and effect of genetic background on phenotype will be discussed.

Conclusion: There is little gene redundancy in mammals; knockout phenotypes exist even if none are immediately apparent; and investigating phenotypes in colonies of mixed genetic background may reveal not only more phenotypes, but also may lead to better understanding of the molecular or cellular mechanism underlying the phenotype and to discovery of modifier gene(s).

One often hears the comment that genetically engineered mice, especially knockout mice, are not useful because they frequently do not yield the expected phenotype, or they don't seem to have any phenotype. These expectations are often based on years of work, and in some instances, thousands of publications of mostly in vitro studies. Examples of unexpected phenotypes, based largely on experience with transforming growth factor beta (*Tgfb*) and basic fibroblast growth factor (*Fgf2*) knockout and transgenic mice, will be presented to discuss possible reasons for unexpected knockout phenotypes. The conclusions will be that the knockout phenotypes do, in fact, provide accurate information concerning gene function, that we should let the unexpected phenotypes lead us to the specific cell, tissue, organ culture, and whole animal experiments that are relevant to the function of the genes in question, and that the absence of phenotype indicates that we have not discovered where or how to look for a phenotype.

Before entering into how one should interpret unexpected knockout phenotypes and how one should deal with lack of knockout phenotypes, it is necessary to give a brief introduction into how knockout mice are made. For detailed information, the following reviews are suggested (1–4). Transgenic technology has had a long history; thus, an introduction to that technology will not be given here. Rather, the following reviews are suggested (5, 6). At this juncture, it should be noted that, although transgenic vertebrates ranging from fish to bovines have been produced, knockout technology has

to date been successful only in mice, even though embryonic stem (ES) cells have been produced from several other species, including hamster (7), rat (8), rabbit (9, 10), pig (11–13), bovine (14, 15), and zebrafish (16). Consequently, the entire discussion will be focused on mice.

Knockout mice are generated by the injection of genetically engineered or gene-targeted ES cells into a mouse blastocyst to generate a chimeric embryo, which in turn can pass on the engineered gene to its offspring. ES cell lines are established from the inner cell mass of a mouse blastocyst, so that when injected into blastocysts, the ES cells can incorporate into the inner cell mass of the recipient blastocysts thereby chimerizing them. Subsequent to transfer of the chimeric blastocysts into uteri of pseudopregnant mice, chimeric mice are born. If the germline of a chimeric mouse is colonized by cells derived from the injected ES cells, the chimera is termed a "germline" chimera. Some of the offspring of the germline chimeras will then carry the engineered gene in their genomes. Gene targeting in ES cells uses the ES cells' DNA repair apparatus to bring about homologous recombination between an exogenous DNA fragment transfected into the ES cell and its homologous region in the genome. Homologous recombination usually results in replacement of the endogenous region with the exogenous fragment, thereby altering the endogenous gene in a prespecified manner. There are many variations on this procedure by which genes can be altered not only to ablate function, but also to make more subtle mutations (17–19). Such procedures can be used to introduce point mutations, remove specific splicing products, switch isoforms, and humanize genes. In addition, technology has recently been

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developed to make conditional and inducible knockouts in which gene function is ablated either in a developmentally specified tissue (20–22) or in an inducible manner (23–26). These techniques, though exciting, will not be further discussed.

Extensive nonredundancy in the TGF β family: Several thousand cell culture studies on the three mammalian transforming growth factor beta proteins (TGF β s 1, 2, and 3) have implicated these growth and differentiation factors in the function of nearly every cell type studied. Expression studies indicated unique and overlapping expression of the three TGF β s (27, 28). For example, overlapping protein localization was found in all gut epithelia, all layers of the skin, all three muscle types, kidney tubules, lung bronchi, cartilage, and bone (Table 1). Together with the fact that all three TGF β s signal through a common TGF type-II receptor (Figure 1), these data strongly suggest considerable redundancy in function. Consequently, it is surprising that, of the >30 phenotypes of the three *Tgfb* knockout mice that we have described (29–31), none appear to be overlapping (Table 2). These results indicate extensive nonredundancy between TGF β ligands even though there is considerable overlap in expression. Of course, these results do not rule out the possibility of some redundancy in some tissues. Combination of the ligand knockouts would uncover such situations, and it is likely that a few will exist, but 30 non-overlapping phenotypes for three ligands strongly suggests that a vast number of their functions are not redundant.

There are several possible explanations for how there can be so much overlap in ligand expression and yet so much specific ligand function. First, TGF β s are secreted as latent peptides and must be activated before they can bind receptors (32–35). The mechanism by which this extracellular processing occurs is not well understood and may be different for each TGF β . Hence, ligand processing presumably determines some functional specificity for the three TGF β s. Second, there is a third type of TGF β receptor, TGF β R3, that can interact with ligand and receptor types I and II before cytoplasmic signaling can occur, though involvement of TGF β R3 is not essential for signaling (36–38). Association with type III receptors is thought to enhance some TGF β R1 and 2/ligand interactions. Upon ligand binding, the serine/threonine receptor TGF β R2 then associates with and phosphorylates the transmembrane serine/threonine receptor TGF β R1, which in turn initiates a phosphorylation-mediated signaling cascade. Hence, combinatorial receptor/ligand interactions will also determine functional specificity. Third, signaling from TGF β R1 can occur through two cytoplasmic signaling proteins called SMAD2 and 3 (39, 40) and, perhaps, through a third called SMAD5 (41). In addition, SMAD6 and 7 can also interact with the other SMADs to inhibit signaling (42–44). Hence, differential SMAD protein interactions with transcriptional machinery will probably also determine functional specificity for the three TGF β ligands. Finally, there may be several non-transcriptional signaling pathways for TGF β s. For example, we have found that TGF β 1-deficient platelets from *Tgfb1* knockout mice have impaired platelet aggregation that can be restored by incubating isolated platelets with recombinant TGF β 1 (unpublished observations). Because platelets do not have a

Table 1. Protein expression of transforming growth factor beta (TGF β) 1, 2, and 3

Tissue/cell type	TGF β 1	TGF β 2	TGF β 3
Cartilage			
Perichondrium	+++	+	++
Chondrocytes	+	++	++
Bone			
Periosteum	++	-	+
Osteocytes		++	++ ++
Tooth			
Ameloblasts	+	-	+
Odontoblasts	-	++	-
Pulp	+	+++	+
Muscle			
Smooth	+	+	++
Cardiac	+	+	+++
Skeletal	+	++	
Lung			
Bronchi	++	++	++
Alveoli	-	-	-
Blood vessels			
Endothelium	-	-	++
Smooth muscle	+	+	+++
Kidney			
Tubules	++	++	++
Basement membrane	-	+++	-
Adrenal			
Cortex	+++	+++	-
Medulla	-	-	-
Gut			
Esophageal epithelium	+++	+	+
Gastric epithelium	+++	+	+
Intestinal epithelium	++	+	+
Basement membrane	-	+++	-
Muscularis	+	+	++
Liver			
Capsule	-	-	++
Parenchyma	-	-	-
Megakaryocytes	+	-	++
Eye			
Lens epithelium	-	-	-
Lens fibers	+++	+	+
Ear			
Cochlear epithelium	-	+	+++
Basement membrane	-	+++	-
CNS			
Meninges	+	+++	+
Glia	-	++	++
Choroid plexus	-	-	++
Skin			
Periderm	++	+	++
Epidermis	+++	+++	+++
Dermis	+	+++	+
Hair follicles	++	++	+

The polyclonal antibodies used were specific for residues 4–19 of TGF β 1 and 2 and residues 9–20 of TGF β 3. The avidin-biotin system was used for staining. Data obtained from immunohistochemical study of Patton et al. (28). Reproduced from *The Journal of Cell Biology*, 1991, 115:1091–1105, by copyright permission of The Rockefeller University Press.

nucleus, there must exist a signaling pathway that is nontranscriptional. In summary, given the complexities of ligand processing, receptor interactions, and signaling pathways, it becomes clear why redundancy in TGF1, 2, and 3 function has not been detected at the whole animal level, even though there is considerable overlap in expression of *Tgfb* gene family members. Consequently, if other gene families function with similar complexity, it is likely that, in the final analysis, little functional redundancy will be found within gene families.

Two striking examples of apparent functional redundancy are worth considering. The first involves myogenic genes, and the second involves retinoic acid receptors. Contrary to early interpretations, redundancy does not now appear to be

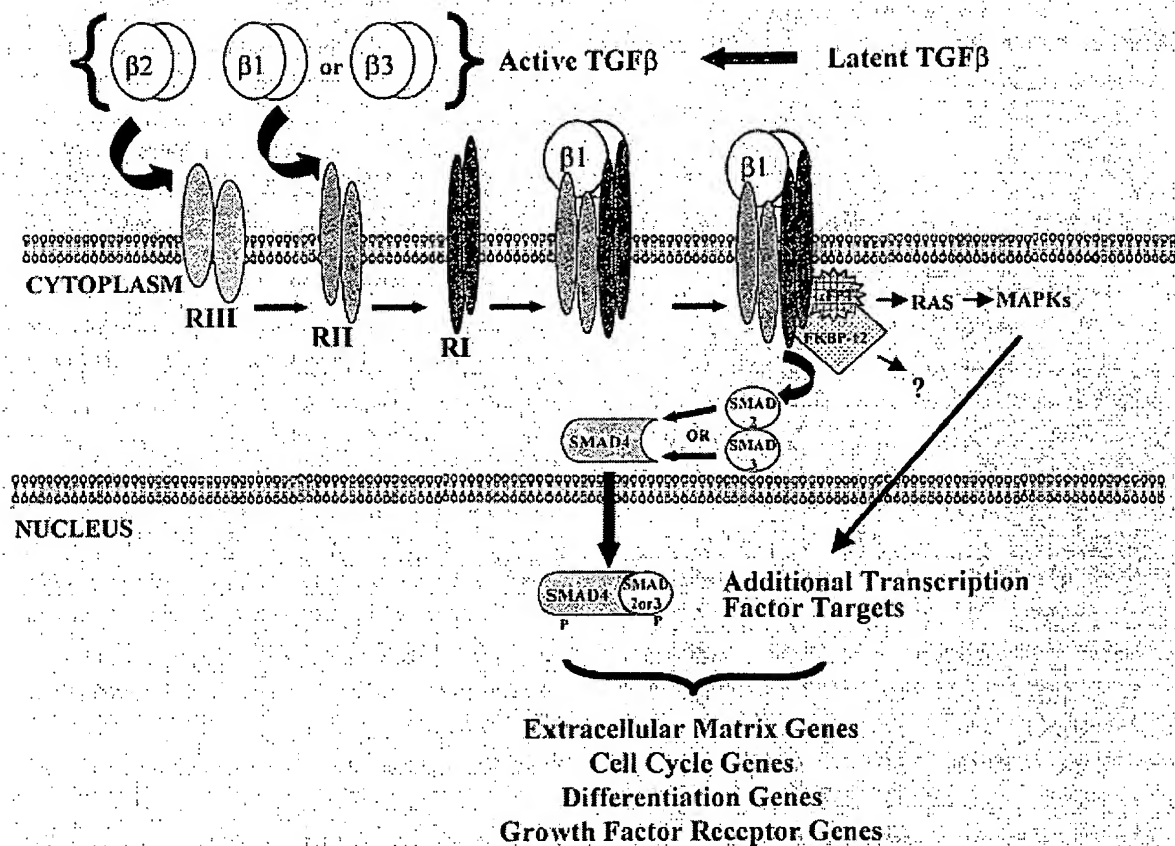


Figure 1. TGFβ signaling pathway. The TGFβ ligands, TGFβ1 (β1), TGFβ2 (β2), and TGFβ3 (β3), exist primarily in a latent form in vivo and are activated by mechanisms not yet clear. In general, TGFβ2 interacts with a TGFβ type III receptor (RIII) before interaction with TGFβ type II (RII) and TGFβ type I (RI) receptors; whereas, the TGFβ1 and TGFβ3 ligands can interact directly with the type II receptor. The ligand receptor complexes can then associate with several cytoplasmic molecules, farnesyl protein transferase (FPT) and FK506 binding protein-12 (FKBP-12), being two potential examples. The receptor-ligand complex signals to the nucleus through threonine/serine phosphorylation of a series of SMAD proteins (related to the *Drosophila* "mothers against decapentaplegic" protein) which then elicit transcriptional regulation of extracellular matrix, cell cycle, differentiation and growth factor receptor genes. The roles of the associated cytoplasmic molecules FPT and FKBP-12 are not clear but are thought to involve RAS pathway signaling and modulation of signaling through the SMAD proteins.

the case for two of the myogenic genes known to be essential for specification of vertebrate skeletal muscle, *Myod* and *Myf5*. Even though the individual knockouts have muscle, and only the combined knockouts do not have muscle (45), it is now clear that each gene functions in the specification of distinct muscle cell lineages. Consequently, in the absence of one source of muscle cells, the other source may compensate for that (46, 47). This should be termed developmental compensation, rather than gene redundancy. On the other hand, with respect to retinoic acid receptors, there is also good evidence for functional redundancy. Similar to the myogenic genes, retinoic acid receptor gene knockout mice have few phenotypes, whereas the combined knockouts have many phenotypes (48, 49). Whether this turns out to be gene redundancy or another case of developmental compensation remains to be determined.

Lack of phenotype: As is the case for TGFβ, there also is a multitude of reports indicating that the FGFs 1 and 2 have important roles in numerous cell types and tissues. Consequently, when the *Fgf2* gene was knocked out by gene targeting, it was quite surprising that there was no obvious phenotype (50). The *Fgf2*^{-/-} animals live a long, healthy life, and fertility and fecundity are normal. Even the pituitary gland, which is the best source of FGF2, appears not to have morphologic defects. The only evidence for any developmental abnormalities is found in hematopoiesis (50), where blood platelet counts are high, and in the cerebral cortex (51, 52), where morphometric analysis reveals decreased cell density. Clearly, these abnormalities are minor, compared with expectations. This was all the more evident because our transgenic mice, in which the human *FGF2* gene was ubiquitously overexpressed by the phosphoglycerate kinase pro-

Table 2. Nonoverlapping phenotypes of *Tgfb1*, 2, and 3 knockout mice and the penetrance of those phenotypes

Knockout mouse phenotype	Penetrance (%) ^a
<i>Tgfb1</i>	
Embryo lethality	50
Preimplantation lethality	50
Yolk sac lethality	50 ^b
Adult phenotypes	50
Multifocal autoimmunity	100 ^c
Platelet defect	100 ^{c,d}
Colon cancer	100 ^{c,d}
Failing heart	100 ^{c,d}
<i>Tgfb2</i> (all perinatal lethality)	
Heart defects	100
Ventricular septum defects	94
Dual outlet right ventricle	19
Dual inlet left ventricle	25
Inner ear defect—lacks spiral limbus	100
Eyes	
Ocular hypercellularity	100
Reduced corneal stroma	100
Urogenital defects in kidney	
Dilated renal pelvis	30
Agnesia (females only)	20
Uterine horn ectopia	40
Testicular ectopia	100
Testis hypoplasia	20
Vas deferens dysgenesis	20
Lung-postnatal	
Dilated conducting airways	100
Collapsed bronchioles	100
Skeletal defects	
Occipital bone	100
Parietal bone	100
Squamous bone	100
Palatine bone (cleft palate)	22
Alisphenoid bone	100
Mandibular defects	100
Short radius and ulna	100
Missing deltoid tuberosity and third trochanter	94
Sternum malformations	25
Rib barreling	94
Rib fusions	13
Spina bifida	100
<i>Tgfb3</i> (perinatal lethality)	
Cleft palate	100

^aSee Table 3 for background dependency of *Tgfb1* knockout phenotypes.

^bDescribed in references 64, 67.

^cRefers to percentage penetrance among animals that survive to birth.

^dUnpublished observations.

Details on the remaining phenotypes can be found in the text and in references 29–31, 63.

motor (53), had very short legs, suggesting an important role of FGF2 in bone development, yet the bones of the knockout animals were normal. This apparent discrepancy between the transgenic and knockout mice indicates that some other FGF signals through the same FGF receptor as does FGF2, and that this other FGF is the true ligand that is important in bone development. Another possibility is that there is "developmental compensation" by alternative mechanisms. In other words, the absence of FGF2 may cause developmental abnormalities during bone development that are then compensated for by another developmental pathway. This alternative would not necessarily require a different FGF to be involved.

After we had made our first analysis of the *Fgf2* knockout mouse and did not find an obvious phenotype, it was easy to explain the "lack of phenotype" by invoking redundancy because there are at least 18 known *Fgf* genes. But in hindsight, it now appears more likely that all members of this large gene family have specific functions, even though they

signal through receptors encoded by only four receptor genes (54). In *Fgf2* knockout mice, evidence was not found for up-regulation of the two ligands most structurally related to FGF2, namely, FGFs 1 and 5 (50). Also, genetic combination of *Fgf2* and *Fgf5* (50) did not reveal redundancy between these similar genes. In addition, further analysis of the mice revealed roles being played in hematopoiesis and vascular tone control (50) as well as in brain development and wound healing (51, 52). Finally, in addition to *Fgf2*, *Fgfs* 3–5, 7, 8 also have been ablated by gene targeting, revealing functions in proliferation of the inner cell mass (*Fgf4*) (55); gastrulation and cardiac, craniofacial, forebrain, midbrain, and cerebellar development (*Fgf8*) (56); brain and inner ear development (*Fgf3*) (57, 58); and two aspects of hair development (*Fgf5* and 7) (59, 60). To date, comparison of *Fgf* knockout phenotypes from 6 of the 18 *Fgf* genes has not turned up overlap except possibly in the cerebellum. Together, these results indicate that each gene has important unique functions. Although a few redundant functions may eventually be found on combination of *Fgf2* with all other *Fgfs* except *Fgf5*, it is clear that 6 of the 18 *Fgf* genes studied by gene targeting have been associated with essentially unique knockout phenotypes.

To summarize, what originally appeared as "lack of phenotype" led many of us to the premature conclusion that other FGFs must have functions redundant to those of FGF2. However, further analysis of *Fgf2* knockout mice has since revealed a wealth of unique functions ranging from thrombocytosis and vascular tone control (50) to brain development and wound healing (51, 52). It is my expectation that further physiologic analysis of the *Fgf2* knockout mouse will reveal functions in the hypertrophic response to hypertension and responses to ischemia/reperfusion injury and bone injury. In the final analysis, it is likely that the major roles of FGF2 may have less to do with getting us to birth than with keeping us alive after birth, whereas several other FGFs clearly have developmental roles.

Effects of genetic background on phenotypic variation: From 100 years of mouse genetics, it has become clear that genetic background plays an important role in the susceptibility of mice to many disorders. Therefore, the phenotypes of knockout mouse strains will also have genetic background dependencies, as was first documented by the Magnuson and Wagner groups (61, 62). The *Tgfb1* knockout mice are an exceptional case in point (Table 3). On a mixed (50:50) 129 x CF1 background (CF1 is a partially outbred strain), about half of *Tgfb1* knockout mice die from a preimplantation developmental defect (63), and the other half die of an autoimmune-like multifocal inflammatory disease at about weaning age (29). If the targeted *Tgfb1* allele is backcrossed onto a C57BL/6 background, 99% of all knockout animals die of the preimplantation defect (63). However, if a *Tgfb1* knockout allele is put onto a mixed 129 x NIH/Ola x C57BL/6 background, embryo lethality is observed during yolk sac development, not during preimplantation development (64). With respect to the multifocal inflammatory disorder of *Tgfb1* knockout mice, if the targeted allele is put onto a 129 x CF1 mixed background (50:50), severe inflammation exists only in the stomach (29); on the mixed 129 x

Table 3. Background dependency of *Tgfb1* knockout phenotypes

Phenotype	Phenotype penetrance on various strains (%)						
	129 x CF1	129 x C57	129 x C3H	C57	129	C3H	129xC57x NIH/Ola
Preimplantation lethality	50	ND	ND	99	ND	ND	0
Yolk sac lethality ^a	0	0	ND	ND	ND	ND	50
Autoimmune disease	50	50	50	1	ND	ND	50
Gastric inflammation	90 ^b	20 ^b	ND	ND	ND	ND	ND
Intestinal inflammation	0	70 ^b	ND	ND	ND	ND	ND
Colon cancer ^c	ND	ND	ND	ND	100	0	ND

Percentage of knockout animals of a given strain that have the designated phenotype.

^aFor details, see references 64, 67.

^bApproximately 10% of animals with autoimmune disease have no detectable gastrointestinal tract inflammation.

^cUnpublished observations.

ND = not determined.

NIH/Ola x C57BL/6 background, the intestines are more severely inflamed than is the stomach (65). Finally, on a predominantly 129 background (129 x CF1; ~97:3), *Tgfb1* knockout mice develop colon cancer if the inflammatory disorder can be eliminated by other genetic manipulations that render the mice immunodeficient (unpublished observations). However, on a predominantly C3H background, immunodeficient *Tgfb1* knockout mice do not develop colon cancer (66). These results suggest that modifier genes exist that can significantly affect the function of TGF β 1 in preimplantation development, yolk sac development, bowel and gastric inflammation, and colon tumor suppression. Progress toward localizing a modifier gene for the yolk sac developmental problem has been made (67).

What is the best genetic background for knockout mice? Because background-dependent phenotypic variability will likely be found for most knockout mice, it will be useful to backcross a targeted allele onto several mouse backgrounds to make congenic strains. In this section, it will be argued that putting a targeted allele on a mixed strain background will also provide useful information. This is not to say that congenic strains are not useful. Rather, the point to be made here is that there also are benefits to looking at mixed strain backgrounds. Again, our experience with *Tgfb* knockout mice will be instructive.

Generating homozygous mutant knockout animals on a mixed genetic background is faster. The ES cells are nearly always from a 129 strain, and the blastocysts into which the targeted ES cells are injected are nearly always C57BL/6. For reasons unknown, this is a good combination for establishing germline transmission of the injected ES cells. The resulting chimeras can then be crossed with any strain desired, but 129, C57BL/6, or Black Swiss mice are most often used, and CF1 mice were used in the case of our *Tgfb1* knockout mice. Heterozygous offspring from this crossing will then be inbred 129 or F1 hybrids of 129 and one of the other strains. Clearly then, the quickest route to having the knockout allele on an inbred strain is through 129. For the other strains several generations of backcrossing is required, which can take well over a year. Unfortunately, strain-129 mice have low fertility and fecundity. Consequently, the number of offspring per litter is usually fewer than six. Although 129 x C57BL/6 hybrids are more robust, upon backcrossing onto C57BL/6, litter size decreases. To the contrary, the Black Swiss and CF1 strains are robust, and litter size often is in excess of 12. The reason for this is probably because they are not truly inbred strains, but

rather are partially outbred through random breeding within their respective strains. Therefore, one of the choices one has is to stay with "pure" genetics at the expense of a lower production rate and considerable delay before generation of experimental animals, or sacrifice some genetic purity to obtain a more efficient production colony. Ideally, one would want to do both, but this often is too expensive.

Mixed genetic background knockout mice often have a wider range of phenotypes. The *Tgfb1* knockout mice backcrossed onto either the 129 or C57BL/6 background (congenics) yield only embryo lethality (63, unpublished observations). On the other hand, when the knockout allele is maintained on mixed genetic backgrounds, embryo and adult phenotypes are maintained.

The *Tgfb2* & *Tgfb3* knockout mice provide further examples. The *Tgfb2* knockout mice have more than two dozen congenital defects and die either immediately preceding or during birth, or within 2 h thereafter (30). Table 2 indicates that most of the phenotypes are only partially penetrant. Though it is not documented, it is likely that the penetrance of some of these phenotypes would increase to nearly 100%, and some of the other phenotypes would disappear were we to put the *Tgfb2* knockout allele on inbred backgrounds. Hence, the mixed strain background probably provided more information than would congenic strains.

The *Tgfb3* knockout mice have a cleft palate (31). One colony of *Tgfb3* knockout mice was left as a mixed background (129 x CF1; 50:50) strain, whereas another colony was backcrossed several generations to the C57BL/6 strain. These two colonies had considerable expressivity differences; the inbred colony had more severe clefting than did the mixed background colony. In the latter, expressivity of clefting varied widely from animal to animal. This variable expressivity within the mixed background colony provided us with the opportunity to obtain far more data on development of the cleft palate and was, therefore, more useful for making assumptions about the cellular and molecular mechanisms by which TGF β 3 supports palate fusion. Hence, using the *Tgfb3* knockout mice, the mixed strain background provided more information than did the congenic strain. Consequently, a wider range of penetrance and expressivity of phenotype is a major advantage of investigating knockout phenotypes in mixed background colonies. Further, variable penetrance of phenotype in a mixed background colony suggests that there are modifier genes for each phenotype that could be obtained by linkage studies.

Conclusions

Questions have been addressed that arose from the last 8 years in which knockout mice have been investigated to analyze gene function at the whole animal level. These questions concern gene redundancy, apparent lack of phenotype in a surprising number of knockout strains, and effects of genetic background on knockout phenotype. Using data obtained principally from *Tgfb* and *Fgf* knockout mice, it is argued that there is probably little redundancy in the genome (i.e., that few genes are dispensable for survival of the species). Apparent lack of phenotype more likely reflects our inability to ask the right questions, or our lack of tools to answer them, than it does a true lack of function. Finally, discussion of genetic background phenotype variability, including variable penetrance and expressivity, was used to present some of the advantages of working with mixed genetic background colonies of knockout mice. For all the examples given here, there are counter examples that must be taken seriously; consequently, these arguments must not be taken as absolutes. For example, if a gene in a particular mouse strain has recently been duplicated, it will most likely be redundant. If one is studying tissue rejection in a knockout mouse, the genetic background obviously must be well defined and preferably inbred. Or, if one wants to use the susceptibility of a particular mouse strain to cancer to investigate the function of the knockout gene in progression of that cancer, the knockout allele must be put on that mouse strain. In general, however, when setting up approaches for investigating a new gene knockout mouse, I believe one would be well advised to assume that: there is little gene redundancy in mammals; there are knockout phenotypes even if none are immediately apparent; and investigating phenotypes in mixed genetic background colonies may not only reveal more phenotypes, but may lead to better understanding of the molecular or cellular mechanism underlying the phenotype, and may lead to modifier gene discovery.

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Front cover Human Genome: Reprinted by permission from Nature, International Human Genome Sequencing Consortium, 409:860-921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila*, *Arabidopsis*, Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all." (Gunter Blobel, courtesy of The Rockefeller University; Marie Curie, Keystone Press Agency Inc; Darwin bust, by permission of the President and Council of the Royal Society; Rosalind Franklin, courtesy of Cold Spring Harbor Laboratory Archives; Dorothy Hodgkin, © The Nobel Foundation, 1964; James Joyce, etching by Peter Blake; Robert Johnson, photo booth self-portrait early 1930s, © 1986 Delta Haze Corporation all rights reserved, used by permission; Albert L. Lehninger, (unidentified photographer) courtesy of The Alan Mason Chesney Medical Archives of The Johns Hopkins Medical Institutions; Linus Pauling, from Ava Helen and Linus Pauling Papers, Special Collections, Oregon State University; Nicholas Proulx, courtesy of ArtToday.com; Barbara McClintock, © David Micklos, 1983; Andrei Sakharov, courtesy of Elena Bonner; Frederick Sanger, © The Nobel Foundation, 1958.)

the target gene in a particular place or at a particular time. The most common of these recombination systems called *Cre/lox*, is widely used to engineer gene replacements in mice and in plants (see Figure 5–82). In this case the target gene in ES cells is replaced by a fully functional version of the gene that is flanked by a pair of the short DNA sequences, called *lox* sites, that are recognized by the *Cre* recombinase protein. The transgenic mice that result are phenotypically normal. They are then mated with transgenic mice that express the *Cre* recombinase gene under the control of an inducible promoter. In the specific cells or tissues in which *Cre* is switched on, it catalyzes recombination between the *lox* sequences—excising a target gene and eliminating its activity. Similar recombination systems are used to generate conditional mutants in *Drosophila* (see Figure 21–48).

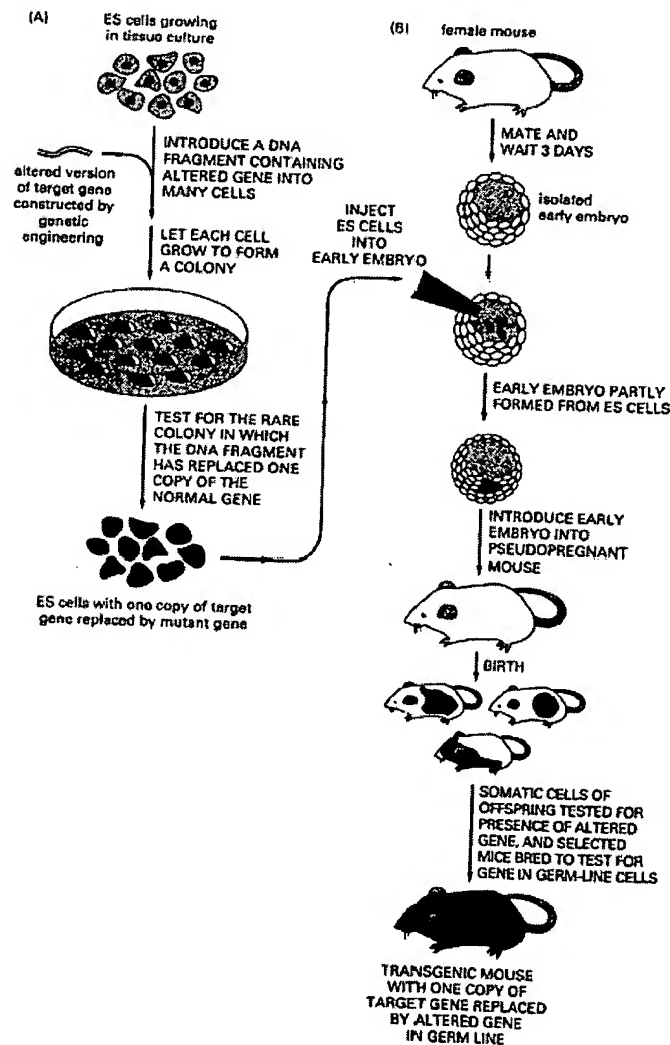


Figure 8–70 Summary of the procedures used for making gene replacements in mice. In the first step (A), an altered version of the gene is introduced into cultured ES (embryonic stem) cells. Only a few rare ES cells will have their corresponding normal genes replaced by the altered gene through a homologous recombination event. Although the procedure is often laborious, these rare cells can be identified and cultured to produce many descendants, each of which carries an altered gene in place of one of its two normal corresponding genes. In the next step of the procedure (B), these altered ES cells are injected into a very early mouse embryo; the cells are incorporated into the growing embryo, and a mouse produced by such an embryo will contain some somatic cells (indicated by orange) that carry the altered gene. Some of these mice will also contain germ-line cells that contain the altered gene. When bred with a normal mouse, some of the progeny of these mice will contain the altered gene in all of their cells. If two such mice are in turn bred (not shown), some of the progeny will contain two altered genes (one on each chromosome) in all of their cells.

If the original gene alteration completely inactivates the function of the gene, these mice are known as knockout mice. When such mice are missing genes that function during development, they often die with specific defects long before they reach adulthood. These defects are carefully analyzed to help decipher the normal function of the missing gene.

Transgenic Plants Are Important for Both Cell Biology and Agriculture

When a plant is damaged, it can often repair itself by a process in which mature differentiated cells "dedifferentiate," proliferate, and then redifferentiate into other cell types. In some circumstances the dedifferentiated cells can even form an apical meristem, which can then give rise to an entire new plant, including gametes. This remarkable plasticity of plant cells can be exploited to generate transgenic plants from cells growing in culture.

When a piece of plant tissue is cultured in a sterile medium containing nutrients and appropriate growth regulators, many of the cells are stimulated to proliferate indefinitely in a disorganized manner, producing a mass of relatively undifferentiated cells called a callus. If the nutrients and growth regulators are carefully manipulated, one can induce the formation of a shoot and then root apical meristems within the callus, and, in many species, a whole new plant can be regenerated.

Callus cultures can also be mechanically dissociated into single cells, which will grow and divide as a suspension culture. In several plants—including tobacco, petunia, carrot, potato, and *Arabidopsis*—a single cell from such a suspension culture can be grown into a small clump (a clone) from which a whole plant can be regenerated. Such a cell, which has the ability to give rise to all parts of the organism, is considered totipotent. Just as mutant mice can be derived by genetic manipulation of embryonic stem cells in culture, so transgenic plants can be created from single totipotent plant cells transfected with DNA in culture (Figure 8-72).

The ability to produce transgenic plants has greatly accelerated progress in many areas of plant cell biology. It has had an important role, for example, in isolating receptors for growth regulators and in analyzing the mechanisms of morphogenesis and of gene expression in plants. It has also opened up many new possibilities in agriculture that could benefit both the farmer and the consumer. It has made it possible, for example, to modify the lipid, starch, and protein storage reserved in seeds, to impart pest and virus resistance to plants, and to create modified plants that tolerate extreme habitats such as salt marshes or water-stressed soil.

Many of the major advances in understanding animal development have come from studies on the fruit fly *Drosophila* and the nematode worm *Caenorhabditis elegans*, which are amenable to extensive genetic analysis as well as to experimental manipulation. Progress in plant developmental biology has, in the past, been relatively slow by comparison. Many of the plants that have proved most amenable to genetic analysis—such as maize and tomato—have long life cycles and very large genomes, making both classical and molecular genetic analysis time-consuming. Increasing attention is consequently being paid to a fast-growing small weed, the common wall cress (*Arabidopsis thaliana*), which has several major advantages as a "model plant" (see Figures 1-46 and 21-107). The relatively small *Arabidopsis* genome was the first plant genome to be completely sequenced.

Large Collections of Tagged Knockouts Provide a Tool for Examining the Function of Every Gene in an Organism

Extensive collaborative efforts are underway to generate comprehensive libraries of mutations in several model organisms, including *S. cerevisiae*, *C. elegans*, *Drosophila*, *Arabidopsis*, and the mouse. The ultimate aim in each case is to produce a collection of mutant strains in which every gene in the organism has either been systematically deleted, or altered such that it can be conditionally disrupted. Collections of this type will provide an invaluable tool for investigating gene function on a genomic scale. In some cases, each of the individual mutants within the collection will sport a distinct molecular tag—a unique DNA sequence designed to make identification of the altered gene rapid and routine.

In *S. cerevisiae*, the task of generating a set of 6000 mutants, each missing



Figure 8-71 Mouse with an engineered defect in fibroblast growth factor 5 (FGF5). FGF5 is a negative regulator of hair formation. In a mouse lacking FGF5 (right), the hair is long compared with its heterozygous littermate (left). Transgenic mice with phenotypes that mimic aspects of a variety of human disorders, including Alzheimer's disease, atherosclerosis, diabetes, cystic fibrosis, and some type of cancers, have been generated. Their study may lead to the development of more effective treatments. (Courtesy of Gail Martin, from J.M. Hebert et al., *Cell* 78:1017-1025, 1994. © Elsevier.)

Genes VII

Benjamin Lewin

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which exogenous DNA is introduced from a bacterium into a host cell. The mechanism resembles that of bacterial conjugation. Expression of the bacterial DNA in its new host changes the phenotype of the cell. In the example of the bacterium *Agrobacterium tumefaciens*, the result is to induce tumor formation by an infected plant cell.

Alterations in the relative proportions of components of the genome during somatic development occur to allow insect larvae to increase the number of copies of certain genes. And the occasional amplification of genes in cultured mammalian cells is indicated by our ability to select variant cells with an increased copy number of some genes. Initiated within the genome, the amplification event can create additional copies of a gene that survive in either intrachromosomal or extrachromosomal form.

When extraneous DNA is introduced into eukaryotic cells, it may give rise to extrachromosomal forms or may be integrated into the genome. The relationship between the extrachromosomal and genomic forms is irregular, depending on chance and to some degree unpredictable events, rather than resembling the regular interchange between free and integrated forms of bacterial plasmids.

Yet, however accomplished, the process may lead to stable change in the genome; following its injection into animal eggs, DNA may even be incorporated into the genome and inherited thereafter as a normal component, sometimes continuing to function. Injected DNA may enter the germline as well as the soma, creating a transgenic animal. The ability to introduce specific genes that function in an appropriate manner could become a major medical technique for curing genetic diseases.

The converse of the introduction of new genes is the ability to disrupt specific endogenous genes. Additional DNA can be introduced within a gene to prevent its expression and to generate a null allele. Breeding from an animal with a null allele can generate a homozygous "knockout", which has no active copy of the gene. This is a powerful method to investigate directly the importance and function of a gene.

Considerable manipulation of DNA sequences therefore is achieved both in authentic situations and by experimental fiat. We are only just beginning to work out the mechanisms that permit the cell to respond to selective pressure by changing its bank of sequences or that allow it to accommodate the intrusion of additional sequences.

The mating pathway is triggered by signal transduction

THE yeast *S. cerevisiae* can propagate happily in either the haploid or diploid condition. Conversion between these states takes place by mating (fusion of haploid spores to give a diploid) and by sporulation (meiosis of diploids to give haploid spores). The ability to engage in these activities is determined by the mating type of the strain.

The properties of the two mating types are summarized in Figure 17.1. We may view them as resting on the teleological proposition that there is no point in mating unless the haploids are of different genetic types; and sporulation is productive only when the diploid is heterozygous and thus can generate recombinants.

The mating type of a (haploid) cell is determined by the genetic information present at the *MAT* locus. Cells that carry the *MAT α* allele at this locus are type α ; like-

wise, cells that carry the *MAT α* allele are type α . Cells of opposite type can mate; cells of the same type cannot.

Recognition of cells of opposite mating type is

Figure 17.1 Mating type controls several activities.

	<i>MATβ</i>	<i>MATα</i>	<i>MATβ/MATα</i>
Cell type	β	α	β/α
Mating	yes	yes	no
Sporulation	no	no	yes
Pheromone	β factor	α factor	none
Receptor	binds α factor	binds β factor	none

Gene Targeting

A Practical Approach

Second Edition

Edited by

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Preface

Over the past ten years it has become possible to make essentially any mutation in the germline of mice by utilizing recombination and embryonic stem (ES) cells. Homologous recombination when applied to altering specific endogenous genes, referred to as gene targeting, provides the highest level of control over producing mutations in cloned genes. When this is combined with site specific recombination, a wide range of mutations can be produced. ES cell lines are remarkable since after being established from a blastocyst, they can be cultured and manipulated relatively easily in vitro and still maintain their ability to step back into a normal developmental program when returned to a pre-implantation embryo. With the exponential increase in the number of genes identified by various genome projects and genetic screens, it has become imperative that efficient methods be developed for determining gene function. Gene targeting in ES cells offers a powerful approach to study gene function in a mammalian organism. Gene trap approaches in ES cells, in particular when they are combined with sophisticated prescreens, offer not only a route to gene discovery, but also to gain information on gene sequence, expression and mutant phenotype.

The basic technology necessary for making designer mutations in mice has become widespread and researchers who have traditionally used cell biology or molecular experiments are adding gene targeting techniques to their repertoire of experimental approaches. A second edition of this book was written for two main reasons. The first was to update previously described techniques and to add new techniques that have greatly expanded the types of mutations that can be made using recombination in ES cells. A chapter in this new edition describes the design and use of site specific recombination for gene targeting approaches and production of conditional mutations. The second reason for the new book was to provide a more in depth discussion of the experimental design considerations that are critical to a successful gene targeting study and to add approaches for analyzing mutant phenotypes, the most interesting part of an experiment. Gene targeting experiments should be designed to go far beyond just making a mutant mouse. The success of a gene targeting experiment no longer lies in the making of the mutation, but depends on the imaginative and insightful analysis of the mutant phenotypes that the mutation provides. A chapter in this edition describes the use of classical genetics in combination with gene targeting to get the most out of a genetic approach to a biological question.

The nature of in vivo gene targeting studies of gene function are such that critical design decisions must be made at every step in the experiment, and each decision can have a major impact on the value of the information obtained. From the start, the type of mutation to be made must be considered

Preface

carefully. Whereas 10 years ago most mutations were designed to create null mutations and were therefore relatively simple to design, at present, a null mutation is only one of a long list of mutations that can be made, each providing different insight into the function of a gene. Point mutations, large deletions, gene exchanges (knock-ins) and conditional mutations are but a few of the choices one faces at the start of a gene targeting experiment. The next choice is the source of DNA for the targeting experiment and ES cell line to be used for the manipulations. Once the mutant ES cell clone has been obtained, there are then a number of alternative approaches that can be used to make ES cell chimeras that depend on the ES cell line which was used. Finally, and most importantly, is the analysis of any phenotype that arises. This second addition discusses techniques used to analyze mutant mice, ranging from standard descriptive evaluation, to a chimera analysis or complicated breeding experiments that utilize double mutants. If mice are simply considered as a 'bag of cells' or an *in vivo* source of selected cell types, then the tremendous resource which mice offer as a model organism is not being realized. The life of a mouse represents a continuum of dynamic processes, including pattern formation, organ development, learning, homeostasis and disease. By making genetic alterations in mice using gene targeting and ES cells, the effects of a given change can be studied in the context of the whole organism.

My goal in editing this book was to provide a manual that could take a newcomer to the exciting field of gene targeting and mutant analysis in mice from a cloned gene to a basic understanding of the genetic approaches available using ES cells, and how each technique can be used to design a particular *in vivo* test of gene function. The book should also provide a valuable bench side resource for anyone carrying out gene targeting or gene trap experiments, a chimera analysis or classical genetic approaches. I would once again like to extend many thanks and my deepest appreciation to all the authors for their great efforts in including detailed protocols and lucid discussions of the various approaches presented. I would also like to thank my family for their strong support and laboratory members past and present for helping to make gene targeting a reality. Finally, since many of the techniques use mice, the experiments should be carried out in accordance with local regulations.

New York, NY

A.L.J.

Gene targeting, principles, and practice in mammalian cells

PAUL HASTY, ALEJANDRO ABUIN, and ALLAN BRADLEY

1. Introduction

When a fragment of genomic DNA is introduced into a mammalian cell it can locate and recombine with the endogenous homologous sequences. This type of homologous recombination, known as gene targeting, is the subject of this chapter. Gene targeting has been widely used, particularly in mouse embryonic stem (ES) cells, to make a variety of mutations in many different loci so that the phenotypic consequences of specific genetic modifications can be assessed in the organism.

The first experimental evidence for the occurrence of gene targeting in mammalian cells was made using a fibroblast cell line with a selectable artificial locus by Lin *et al.* (1), and was subsequently demonstrated to occur at the endogenous β -globin gene by Smithies *et al.* in erythroleukaemia cells (2). In general, the frequencies of gene targeting in mammalian cells are relatively low compared to yeast cells and this is probably related to, at least in part, a competing pathway: efficient integration of the transfected DNA into a random chromosomal site. The relative ratio of targeted to random integration events will determine the ease with which targeted clones are identified in a gene targeting experiment. This chapter details aspects of vector design which can determine the efficiency of recombination, the type of mutation that may be generated in the target locus, as well as the selection and screening strategies which can be used to identify clones of ES cells with the desired targeted modification. Since the most common experimental strategy is to ablate the function of a target gene (*null allele*) by introducing a selectable marker gene, we initially describe the vectors and the selection schemes which are helpful in the identification of recombinant clones (Sections 2–5). In Section 6, we describe the vectors and additional considerations for generating subtle mutations in a target locus devoid of any exogenous sequences. Finally, Section 7 is dedicated to the use of gene targeting as a method to express exogenous genes from specific endogenous regulatory elements *in vivo*, also known as 'knock-in' strategies.

enrich populations of transfected cells for targeted integration events (Section 4.2.1).

2.1 Design considerations of a replacement vector

The principal consideration in the design of a replacement vector, is the type of mutation generated. Secondary (yet still important) considerations relate to the selection scheme and screening techniques required to isolate the recombinant clones. The recombinant alleles generated by replacement vectors typically have a selection cassette inserted into a coding exon or replacing part of the locus. It is important to consider that, exon interruptions and small deletions will not necessarily ablate the function of the target gene to generate a null allele. Consequently, it is necessary to confirm that the allele which has been generated is null by RNA and/or protein analysis and in many cases transcripts and truncated proteins from such a mutant allele can be detected. Considering that products from the mutated locus may have some function (normal or abnormal) it is important to design a replacement vector so that the targeted allele is null, particularly in the absence of a good assay for the gene product. Disruption or deletion of the coding sequence by the positive selection marker will in most instances ablate a gene's function. However in some situations a truncated protein may be generated which retains some biological activity, thus some knowledge of mutations in a related gene in another organism can be helpful in the determination of the possible function of a targeted allele. Null alleles are more likely to occur by deleting or recombining a selection cassette into more 5' exons rather than exons that encode the C-terminus of the protein, since under these circumstances minimal portions of the wild-type polypeptide would be made.

There are several considerations to take into account when a positive selection marker is to be inserted into an exon. One critical consideration is that since the length of an exon can influence RNA splicing (3), an artificially large exon caused by the insertion of a selectable marker may not be recognized by the splicing machinery and could be skipped. Thus, transcripts initiated from the endogenous promoter may delete the mutated exon from the mRNA species or even additional exons. If a skipped exon is a coding exon whose nucleotide length is not a multiple of three (codon) the net result will be both a deletion and a frame-shift mutation of the gene, which will often generate a null allele. However, if the disrupted coding exon has a nucleotide length which is a multiple of three, if spliced out, this would result in a protein with a small in-frame deletion which may retain partial or complete function. The same concept applies to gene targeting vectors in which exons are being deleted and replaced by the selectable marker. Deletion of an exon or group of exons with a unit number of codons may also result in a functional protein product with an in-frame deletion. For most purposes it is advisable to delete portions or all of the target gene so that the genetic

Production of targeted embryonic stem cell clones

MICHAEL P. MATISE, WOJTEK AUERBACH and ALEXANDRA L. JOYNER

1. Introduction

The discovery that cloned DNA introduced into tissue culture cells can undergo homologous recombination at specific chromosomal loci has revolutionized our ability to study gene function in cell culture and *in vivo*. In theory, this technique, termed gene targeting, allows one to generate any type of mutation in any cloned gene. The kinds of mutations that can be created include null mutations, point mutations, deletions of specific functional domains, exchanges of functional domains from related genes, and gain-of-function mutations in which exogenous cDNA sequences are inserted adjacent to endogenous regulatory sequences. In principle, such specific genetic alterations can be made in any cell line growing in culture. However, not all cell types can be maintained in culture under the conditions necessary for transfection and selection. Over ten years ago, pluripotent embryonic stem (ES) cells derived from the inner cell mass (ICM) of mouse blastocyst stage embryos were isolated and conditions defined for their propagation and maintenance in culture (1, 2). ES cells resemble ICM cells in many respects, including their ability to contribute to all embryonic tissues in chimeric mice. Using stringent culture conditions, the embryonic developmental potential of ES cells can be maintained following genetic manipulations and after many passages *in vitro*. Furthermore, permanent mouse lines carrying genetic alterations introduced into ES cells can be obtained by transmitting the mutation through the germline by generating ES cell chimeras (described in Chapters 4 and 5). Thus, applying gene targeting technology to ES cells in culture affords researchers the opportunity to modify endogenous genes and study their function *in vivo*. In initial studies, one of the main challenges of gene targeting was to distinguish the rare homologous recombination events from more commonly occurring random integrations (discussed in Chapter 1). However, advances in cell culture and in selection schemes, in vector construction using isogenic DNA, and in the application of rapid screening procedures have made it possible to identify homologous recombination events efficiently.

What's Wrong With My Mouse?

*Behavioral Phenotyping of
Transgenic and Knockout Mice*

Jacqueline N. Crawley, Ph.D.



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Preface

Targeted mutation of genes expressed in the nervous system is an exciting new research field that is forging a remarkable amalgam of molecular genetics and behavioral neuroscience. My laboratory in Bethesda has been the fortunate recipient of visits from many molecular geneticists over the past five years, who come to ask, "What's wrong with my mouse? Can you tell us what behaviors are abnormal in our null mutants? And how do you measure behavior, anyway?"

We have had some remarkable opportunities to collaborate with outstanding molecular geneticists in the National Institutes of Health Intramural Research Program and throughout the world on investigations of the behavioral effects of mutations in genes expressed in the mouse brain. Each of these collaborations has been a learning experience, increasing our understanding of the optimal experimental design for analyzing behavioral phenotypes of mutant mice. What are the best tests to address each specific hypothesis? Which methods work best for mice? Which rat tasks can be adapted for mice? What are the correct controls? What are the hidden pitfalls, lurking artifacts, false positives, and false negatives? Which statistical tests are most sensitive for detection of the genotype effect? What is the minimum number of animals necessary for each genotype, gender, and age? Our laboratory and many others are gradually working out the best methods for behavioral phenotyping of transgenic and knockout mice.

In the same conversations, molecular geneticists frequently asked me to recommend a book they could consult to learn more about behavioral tests for mice. Apparently the scientific book publishers are receiving similar queries. Ann Boyle and Robert Harrington at John Wiley & Sons, convinced of a real need for such a book, sweet-talked me into filling the void. *What's Wrong With My Mouse?* is written for these pioneering molecular geneticists, and for the talented students who will be the next driving force in moving the field forward.

On a personal level, I would like to express deep appreciation to all of my behavioral neuroscientist colleagues around the world for their outstanding work, past, present, and future. Your contributions to the excellence and abundance of mouse behavioral tests provide

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x PREFACE

the foundation for the rapidly expanding scientific discoveries forthcoming from behavioral phenotyping studies of transgenic and knockout mice. This book is a testament to your accomplishments.

JACQUELINE N. CRAWLEY, Ph.D.
Chevy Chase, Maryland

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Designer Mice

The disease is inherited. Family pedigrees indicate an autosomal dominant gene. Linkage analyses reveal one strongly associated chromosomal locus. Mapping identifies the gene. The cDNA for the gene is sequenced. The anatomical distribution of the gene is primarily in the brain. The symptoms of the disease are primarily neuropsychiatric. There is no treatment for the disease. The disease is lethal.

Your mission, should you choose to accept it, is to develop a treatment for the disease. Replacement gene therapy is the best hope. But you don't know the gene product, you don't know its function, and you don't know if gene delivery would be therapeutic. Where do you start?

These days, you may choose to start with a targeted gene mutation, to generate a mutant mouse model of the hereditary disease. A DNA construct containing the mutated form of the responsible gene is developed. The construct is inserted into the mouse genome. A line of mice with the mutated gene is generated. Characteristics of the mutant mice are identified in comparison to normal controls. Salient characteristics relevant to the human disease are quantitated. These diseaselike traits are then used as test variables for evaluating the effectiveness of treatments. Putative treatments are administered to the mutant mice. A treatment that prevents or reverses the disease traits in the mutant mice is taken forward for further testing as a potential therapeutic treatment for the human genetic disease. Gene therapy, based on targeted gene replacement of the missing or incorrect gene in the human hereditary disease, is described in Chapter 12. In the future, medicine may shift emphasis from treating the symptoms to administering replacement genes that effectively and permanently cure the disease.

Targeted gene mutation in mice represents a new technology that is revolutionizing biomedical research. Transgenic mice have an *extra gene* added. An additional copy of a normal gene is inserted into the mouse genome to study overexpression of the gene product. Or a new gene is added that is not normally present in the mouse genome. The new gene may be the aberrant form of a human gene linked to a disease. For example, the mutated form of the human *huntingtin* gene is added to the mouse genome to generate a mouse model of Huntington's disease. Knockout mice have a *gene deleted*. The null mutant homozygous

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knockout mouse is deficient in both alleles of a gene; the heterozygote is deficient in one of its two alleles for the gene. The genotype is $-/-$ for the null mutant, $+/-$ for the heterozygote, and $+/+$ for the wildtype normal control. The phenotype is the set of observed characteristics resulting from the mutation. Phenotypes include biochemical, anatomical, physiological, and behavioral characteristics.

Targeted mutations of genes expressed in the brain are revealing the mechanisms underlying normal behavior and behavioral abnormalities. Mouse models of human neuropsychiatric diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, obesity, anorexia, depression, alcoholism, drug addiction, schizophrenia, and anxiety, are likely to be characterized by their behavioral phenotype.

This book is designed to introduce the novice to the rich literature of behavioral tests in mice and to show how to optimize the application of these tests for behavioral phenotyping of mutant mice. Based on our experiences, our laboratory is working toward a unified approach for the optimal conduct of behavioral phenotyping experiments in mutant mice. Recommendations are offered for a three-tiered sequence of behavioral tests, applicable to each behavioral domain relevant to genes expressed in the mammalian brain.

SCOPE

This book is designed as an overview of the mutant mouse technology and an introduction to the field of behavioral neuroscience, as it can be applied to behavioral phenotyping of transgenic and knockout mice. Molecular geneticists may browse through the chapters relevant to their gene, to get ideas for possible tests to try. Behavioral neuroscientists who have no experience with mutant mice may wish to read about the methods for developing a transgenic or knockout, the behavioral tests that have been effectively applied, and some of the successful experiments published in the genetics literature.

Chapters are organized around behavioral domains, including general health, neurological reflexes, developmental milestones, motor functions, sensory abilities, learning and memory, feeding, sexual and parental behaviors, social behaviors, and rodent paradigms relevant to fear, anxiety, depression, schizophrenia, reward, and drug addiction. Each chapter begins with a brief history of the early work in the field and the present hypotheses about mechanisms underlying the expression of the behavior. A list of general review articles and books is offered for each topic, encouraging the interested reader to gain more in-depth knowledge of the relevant literature.

Standard tests are then presented in detail. Highlighted are those tasks that have been extensively validated in mice. Demonstrations of genetic components of task performance are described, including experiments comparing inbred strains of mice (strain distributions), quantitative trait loci approaches (linkage analysis), and naturally occurring mutants (spontaneous mutations). Experimental design and specific behavioral tasks are presented as simply as possible. Extensive references are included for each behavioral test to obtain more complete methods from the primary experimental literature on the topic.

Illustrations are provided for the most frequently used behavioral tasks. Photographs of the equipment or diagrams of the task accompany the text. Samples of data are shown. The data presentation is designed to indicate the qualitative and quantitative results that can be expected when the task is properly conducted.

Each chapter includes the results of several representative experiments in which these tasks are successfully applied to characterize transgenic and knockout mice. Examples are

WHAT'S WRONG WITH MY MOUSE



Transgenic and knockout mutations provide an important means for understanding gene function, as well as for developing therapies for genetic diseases. This engaging and informative book discusses the many advances in the field of transgenic technology that have enabled researchers to bring about various changes in the mouse genome. Equal emphasis is given to both the principles of transgenic and knockout methods and their applications. A clear and concise format provides researchers with a comprehensive review of the behavioral paradigms appropriate for analyzing mouse phenotypes.

What's Wrong with My Mouse? explains the differences between transgenic knockout mice and their wild-type controls, while providing critical information about gene function and expression. This volume recognizes that newly identified genes can provide useful insights into brain functioning, including brain malfunctioning in disease states. Written by a world-renowned expert in the field, the material also covers:

- How to generate a transgenic or knockout mouse
- Motor functions (open field, holeboard, rotarod, balance, grip, circadian activity, etc.)
- Sensory abilities (olfaction, vision, hearing, taste, touch, nociception)
- Reproductive behavior, social behavior, and emotional behavior

Researchers in neuroscience, pharmacology, genetics, developmental biology, and cell biology will all find this book essential reading.

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